Effects of gabexate mesylate on the gut microbiota and metabolomics in rats with sepsis

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Abstract

**Background:** Sepsis develops in millions of people worldwide annually. Only 30%–40% of septic shock cases have positive microbial culture results\(^1\). Currently, there is no clear early diagnostic marker for sepsis. The gut microbiota and metabolomics significantly change in sepsis. Previous studies have reported that gabexate mesylate (GM) could alleviate liver and lung injury and high inflammatory status in sepsis. This study aimed to examine the changes in gut microbiota and metabolomics after GM application.

**Methods:** Sixty Sprague–Dawley (SD) rats were randomly divided into the sham control group (SC), sepsis after cecal ligation and puncture (CLP) group, and intraperitoneal GM injection 1 h after CLP (GM) group. Colonic feces were collected to examine the gut microbiota and metabolism using 16SrRNA and liquid chromatography-mass spectrometry, and bioinformatics methods were performed to analyze the microbiome and metabolomics. The mortality rate of the three groups was measured 24 h after the procedure. Moreover, lung tissues were collected for hematoxylin-eosin (HE) staining.

**Result:** 1. In the comparison of the SC and GM groups, the relative abundance of Firmicutes and ratio of Firmicutes to Proteobacteria in the CLP group decreased at the phylum level, and the Proteobacteria increased. The Lactobacillus at the genus level of the CLP group significantly decreased, while the Escherichia–Shigella and Akkermansia increased. The α-diversity and β-diversity of the CLP and SC groups were significantly different. The principal coordinate analysis showed that the microbiota in the SC and GM groups were similar and had a large difference from that of the CLP group. Linear discriminant analysis effect size analysis showed that the different genera of the CLP and SC groups were Lactobacillus, Romboutsia, and Escherichia–Shigella. The different genera of the CLP and GM groups were Lactobacillus, Pygmaiobacter, Erysipelotrichaceae_UCG_003, Escherichia–Shigella, and Akkermansia.

2. Principal component analysis indicated that the metabolic profiles of SC and GM groups were close to each other and significantly separated from the CLP group. Orthogonal partial least squares discriminant analysis showed that CLP and SC groups and CLP and GM groups could be separated into different groups because of metabolic differences. It was found that GM mainly regulates sphingolipid metabolism, histidine metabolism, steroid biosynthesis, glycerophospholipid metabolism, primary bile acid biosynthesis, and other metabolic pathways.

3. Combined analysis of differential gut microbiota and metabolites showed that CLP-rich genera (Akkermansia, Escherichia–Shigella, and Proteus) were positively associated with LysoPE (15:0/0:0) and quinolinic acid, and GM-rich genus (Lactobacillus) was negatively correlated with LysoPE (15:0/0:0) and quinolinic acid. CLP-rich genera (Akkermansia, Escherichia–Shigella, and Proteus) were negatively correlated with leukotriene B4 and 9,10-DHOME, and GM-rich genus (Lactobacillus) was positively correlated with leukotriene B4 and 9,10-DHOME.
4. The mortality rate in the CLP group was significantly higher than those in the SC and GM groups. HE staining showed that inflammatory infiltration, edema, and structural damage of the lung tissues in the CLP group were more severe than those in the SC and GM groups.

**Conclusion:** GM could affect the changes in Lactobacillus, Akkermansia, Escherichia–Shigella, and other genera; regulate the relative abundance of LysoPE (15:0/0:0), quinolinic acid, leukotriene B4, 9,10-DHOME, and other differential metabolites; and attenuate acute lung injury caused by sepsis, which may provide a potential mechanism for GM application in sepsis treatment.

**Introduction**

Sepsis affects the lives and health of millions of people worldwide annually. It is a common fatal and costly disease. Sepsis results in life-threatening organ dysfunction caused by loss of control over infection[2]. One of the main causes of organ failure in sepsis is the inflammatory response due to infection[3]. According to statistical data, the sepsis mortality rate in hospitalized patients is 26.7%, and the sepsis mortality rate in the intensive care unit is 41.9%[4]. Sepsis causes severe immune dysfunction and chronic critical disease characterized by catabolism, usually associated with prolonged inflammation, immunosuppression, organ damage, and muscle atrophy[5].

The gut is the main origin of microorganisms that lead to sepsis. The gut microbiota could prevent the colonization of intestinal pathogens, but the impaired gastrointestinal function and antibiotic administration in sepsis cause significant changes in the gut microbiota, manifested as dysbacteriosis[6, 7]. Decreased diversity of the microbiome[8], thinning of the gut mucus layer and increased gut permeability, allow pathogens to thrive in the gut lumen[9]. Several studies reported that the gut microbiota is an important environmental factor in sepsis development[10, 11], and dysbiosis increases susceptibility to sepsis[12]. Significant changes in the specific microbiome may be associated with sepsis progression[13]. Gut bacterial content determines the severity of systemic damage in sepsis, suggesting that dysbiosis also affects sepsis prognosis[14]. Increasing evidence indicates that gut microbiota plays a key role in pulmonary immunity and is associated with airway homeostasis[15].

The metabolome is the collection of small molecule metabolites produced by the body's metabolic processes[16, 17]. It also reflects the situation of metabolites at a specific time during the disease process[18]. Unlike genomics, transcriptomics and proteomics, metabolomics, as a direct biomarker of biochemical activity, are more likely to directly correlate with phenotype[19]. There are two approaches to studying the metabolome, both targeted and untargeted. Untargeted metabolomics uses unbiased screening methods to identify thousands of metabolites, enabling the detection of unknown metabolites[20]. Compared with nuclear magnetic resonance spectrometry or gas chromatography-mass spectrometry, it is more difficult to identify metabolites with liquid chromatography-mass spectrometry (LC-MS)[21]. However, it has the advantages of high sensitivity and wide detection range of metabolites. The gut microbiota plays a key role in sepsis and septic shock, and gut dysbiosis could contribute to the onset
of sepsis by producing harmful metabolites and altering host physiological processes. By integrating host and environmental factors, 16S rRNA and metabolomic studies could explore the overall changes in the gut microbiota and metabolic disturbances involved in gut microbiota–host interactions.

Gabexate mesylate (GM) is a non-peptidic proteolytic enzyme inhibitor with potent inhibition of platelet aggregation and renin formation to prevent disseminated intravascular coagulation\(^{[22,23]}\). Anti- and pro-inflammatory imbalance occurs, and levels of tumor necrosis factor-a (TNF-a)\(^{[24]}\), interleukin-6 (IL-6), and high mobility group box 1 (HMGB-1) increase, leading to acute lung injury\(^{[25]}\). Several studies reported that pretreatment with GM significantly reduced serum TNF-a, IL-6, thrombin-antithrombin complex, and HMGB-1 levels\(^{[26–28]}\), and alleviated lipopolysaccharide-induced lung injury in rats with sepsis\(^{[29]}\). A case report shows that GM reverses amylase, lipase, and white blood cell counts in patients with septic shock and improves clinical outcomes\(^{[30]}\).

We hypothesized that GM could ameliorate sepsis-induced lung damage and alter the gut microbiota and metabolome. By comparing the changes in the gut microbiota and metabolome in the SC, CLP, and GM groups, the correlation analysis revealed the relationship between differential microbiota and metabolites after GM treatment in the rats with sepsis.

### Materials And Methods

The following instruments and reagents were used: bench-type high-speed centrifuge (Eppendorf), PCR instrument (Bio-Rad), electrophoresis instrument (Tanon), Illumina NovaSeq sequencer (Illumina), Vanquish ultra-high-performance liquid chromatograph (Thermo Fisher Scientific), high-resolution mass spectrometry Q Exactive HFX (Thermo Fisher Scientific), Column ACQUITY UPLC HSS T3 (2.1 mm × 100 mm,1.7 μm) (Waters); Magnetic Bead Method Soil and Fecal DNA Extraction Kit (Tiangen Biochemical Technology [Beijing] Co., Ltd.), Phusion High-Fidelity PCR Master Mix with GC Buffer (Thermo Fisher Scientific), TruSeq DNA PCR-Free Sample Preparation Kit (Illumina), methanol (CNW Technologies), acetonitrile (CNW Technologies), ammonium acetate (Sigma-Aldrich), ammonia (Fisher Chemical), and Ultra Pure Water (Watsons).

#### Experimental animals and grouping

Sixty 6–8-week-old adult male Sprague–Dawley (SD) rats weighing 200–250 g (https://www.vitalriver.com/). All rats were housed in a 12/12-h day and night animal room for a week before the experiment and allowed to eat and drink ad libitum. The temperature in the animal room was controlled from 20 °C to 25 °C, and the relative humidity was controlled from 40% to 60%. SD rats were randomly divided into sham operation control (SC) group, sepsis after cecal ligation and puncture (CLP) group, and intraperitoneal injection of GM (0.1 g, Changzhou Siyao Pharmaceuticals Co., Ltd.) 1 h after CLP group. The establishment of the CLP model was based on our previous studies\(^{[31,32]}\). The rats were weighed and anesthetized by intraperitoneal injection of 30 mg/kg pentobarbital. A 2-cm incision was made on the midline of the abdomen, the cecum was exposed, and the feces was squeezed in the cecum.
to the free end. It was ligated at the middle and lower one-third of the cecum, and an 18-G needle was used to puncture the middle of the cecal ligation. After the puncture, a small amount of feces is squeezed out, and the cecum is returned to the original position of the abdomen. The muscle and skin layer were sutured with No. 5 and No. 3 surgical sutures, respectively. Immediately after the procedure, subcutaneous injection of 50 mL/kg of warm saline was used for resuscitation, and the rats were irradiated with warm light for 30 min. The SC group underwent the same procedure without ligation and puncture. The GM group received intraperitoneal injection of GM (40 mg/kg) 1 h after CLP. One hour after CLP, rats in the SC and CLP groups were intraperitoneally injected with normal saline. The experiments were conducted according to the guidelines established by the National Institutes of Health (http://grants1.nih.gov/grants/olaw/) and approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

**Sample collection**

The survival rate of the rats was determined 24 h after the operation. Colonic feces of the surviving rats in each group (two tubes of samples were obtained from each rat) were squeezed and placed into 2-mL cryopreservation tubes separately and stored in liquid nitrogen. The perfused lung tissues were collected, and the perfusion procedure was performed similar to that in the previous study. Lung tissues were fixed with 4% paraformaldehyde, embedded in paraffin overnight, stained with hematoxylin and eosin (HE), and observed under a microscope (Olympus, Japan).

**Fecal DNA extraction and high-throughput 16S rRNA sequencing**

DNA was extracted from the fecal samples using sodium dodecyl sulfate; then, the DNA was diluted to 1 ng/μL sterile water, and the primers were 314F:CCTAYGGGRBGCASCAG and 806R:GGACTACNNGGGTATCTAAT, targeting the 16S V3–V4 region. The DNA purity and concentration were verified by electrophoresis in 2% agarose gel and purified by magnetic spheres. Library construction was qualified and sequenced on NovaSeq 6000.

**16S rRNA sequencing data analysis**

The original data were spliced and filtered to obtain valid data. Based on the valid data, the clusters of operating taxonomic units (OTUs) and species classification were analyzed with 97% consistency. Subsequently, the species was recorded on the representative sequence of each OTU to obtain species-related information and species-based abundance distribution. Chao1, ACE, Shannon, Simpson, Good's coverage, and phylogenetic diversity (PD) whole tree indices were calculated using QIIME software. Principal component analysis (PCA) was performed using the ade4 package and ggplot2 package of R software (version 2.15.3); principal coordinate analysis (PCoA) was performed using WGCNA and stats and ggplot2 packages; and R software was used to draw the rarefaction curve and analyze the differences between the groups of α- and β-diversity. Parametric and nonparametric tests were used in the data analysis, t-test and Wilcoxon test were used for two-group comparison, and Tukey's and Wilcoxon tests were used for three-group comparison. Linear discriminant analysis (LDA) effect size (LEfSe) was
performed by comparing two or more groups and examining markers that are statistically different between groups. LEfSe used the Kruskal–Wallis rank-sum test to detect characteristics with significantly different abundance levels among the specified taxa, and LDA was performed to estimate the effect size, emphasizing statistical significance and biological relevance. Tax4Fun was used to predict the microbiota function. Correlations between different species and functional pathways were calculated using Spearman rank correlations, and correlations were presented using a heatmap.

**Fecal sample preparation for metabolomic analysis**

Moreover, 25 mg of thawed stool sample was obtained and mixed with 500 μL of extract (methanol:acetonitrile:water = 2:2:1). Then, 20 μL of 0.3 mg/mL 2-chloro-D-phenylalanine was added. All samples were ground and ultrasonically extracted in ice water for 5 min, and the steps were repeated two to three times and allowed to stand at −40 °C for 1 h. Then, the supernatant was used in the analysis by centrifugation at 12,000 rpm for 15 min at 4 °C. Each 20 μL of sample was mixed to obtain a quality control (QC) sample, and 200 μL of QC sample was collected for QC analysis.

**LC-MS metabolomics-based data acquisition**

Chromatographic separation of metabolites was performed using ultra-high-performance liquid chromatography (UPLC) and UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) (autosampler temperature, 4°C; injection volume, 2 μL). Liquid chromatography phase A consisted of an aqueous phase containing 25 mmol/L ammonium acetate and 25 mmol/L ammonia water, and phase B contained acetonitrile. The conditions of the ionization source of the electrospray were set as follows: the flow rate of the gas in the sheath is 30 Arb, the flow rate of the auxiliary gas is 25 Arb, and the capillary temperature is 350 °C. The resolution of mass spectrometry for primary information acquisition was set to 60000, and secondary information acquisition was set to 7500. The collision energy was 10/30/60 in normalized collision energy mode, and the spray voltage was 3.6 kV (positive) or −3.2 kV (negative). The Thermo Q Exactive HFX mass spectrometer collects mass spectral data as raw data.

**Metabolomics data analysis**

After processing the raw data for peak identification, peak extraction, peak alignment, and integration, a dataset containing mass-to-charge ratio, retention time, and peak intensity was obtained. Then, the data were preprocessed and normalized and matched with an internal mass spectrometry database (BiotreeDB V2.1), and metabolites were annotated with a cutoff point of 0.3. Unsupervised PCA was performed to visualize metabolic changes between groups. Discriminant analysis of orthogonal partial least squares (OPLS-DA) was applied to visualize the maximum difference between the two groups. The variable importance in projection (VIP) was calculated. A high VIP indicated that the metabolite has a large contribution to the grouping. Data were analyzed using one-way analysis of variance and Tukey’s post hoc test (SPSS software version 21.0, Chicago, IL). Metabolites with VIP > 1 and P < 0.05 were selected as differential metabolites. Heatmaps were used to show trends in differential metabolites. Pathway analysis
was performed using MetaboAnalyst 3.0, which is based on database sources, including the KEGG database (http://www.genome.jp/kegg/) and Human Metabolome Database (https://hmdb.ca/).

Results

Composition of the gut microbiota in the three groups

The rarefaction curve of each sample was close to saturation, the amount of sequencing data was reasonable, and the sequencing depth was sufficient (Figure 1A). Sample valid sequences with 97% identity were clustered into OTUs. The gut microbiota was annotated with representative OTUs, with a total of 2,126 OTUs. Moreover, 660, 616, and 850 OTUs were identified in the SC, CLP, and GM groups, respectively, of which 495 OTUs were shared by three groups (Figure 1B). In the phylum-level gut microbiota, the predominant phyla in the SC and GM groups were Firmicutes, Proteobacteria, and Verrucomicrobiota in the CLP group (Figures 1C). The Firmicutes–Proteobacteria ratio was significantly decreased in the CLP group (Figures 1D). The predominant genera in the SC and GM groups were Lactobacillus, Ruminococcus, and Blautia. The predominant genera in the CLP group were Escherichia–Shigella, Akkermansia, and Enterococcus (Figure 1E).

α-Diversity analysis

The Simpson index represented the diversity and evenness of species distribution. The Wilcoxon rank-sum test showed that the diversity and evenness (Simpson index) of species distribution in the SC and GM groups were higher than those in the CLP group. The Shannon index, which measures the total number of microbial communities and their proportions, was also higher in the SC and GM groups than in the CLP group. The Chao1 index was used to evaluate the total number of species. The Chao1 index was higher in the SC group than in the CLP and GM groups. ACE was used to estimate the number of OTUs in the community. The ACE was higher in the SC group than in the CLP and GM groups. Good's coverage index represents the sequencing depth. Good's coverage rates of the three groups were all greater than 99.8%, indicating that the sequencing depth of the gut microbiota was ideal. The PD whole tree index represents the genetic relationship of the species in the community. The PD whole tree index in the SC and GM groups was higher than in CLP group (Table 1).

β-Diversity analysis

To assess the degree of similarity between microbial communities, conducted sampling comparative analysis (β-diversity analysis) for different groups. The PCoA results showed that samples from the SC, CLP, and GM groups formed three distinct clusters, among which samples from the SC and GM groups tended to cluster together, indicating that the two groups had similar species composition. The CLP group was far away, indicating that it had a larger community difference compared with the SC and GM groups (Figure 1F).

Differential microbiota analysis in the SC, CLP, and GM groups
To identify the specific microbiota associated with the SC, CLP, and GM groups, we used LEfSe analysis. The LSD score was used to evaluate the influence of the relative abundance of species in different groups on the difference effect (LDA > 4 and $P < 0.05$). There are three differential genera, namely, Lactobacillus, Romboutsia, and Escherichia–Shigella, between the CLP and SC groups (Figure 2A). Similarly, there were five differential genera between the CLP and GM groups, namely, Lactobacillus, Pygmaiohabitum, Erysipelotrichaceae_UCG_003, Escherichia–Shigella, and Akkermansia (Figure 2B). The only differential genera between the SC group and GM group was Parasutterella (Figure 2C).

**Correlation analysis of genera between the CLP and GM groups**

The gut microbiota is interconnected to maintain homeostasis, and studying the interactions between different genera contributes to finding the important role of microbiota of the CLP and GM groups (Spearman correlation coefficients $<-0.80$ or $>0.80$, $P < 0.05$). Notably, the GM-enriched genera had higher associations than the CLP group-enriched genera. Akkermansia was strongly positively correlated with Desulfovibrio. Lachnospiraceae UCG-006 was strongly negatively correlated with Lactobacillus in the CLP group. Moreover, Lactobacillus and Desulfovibrio were strongly negatively correlated (Supplementary Figure 1). In the GM group, Ruminococcus was strongly negatively correlated with Escherichia–Shigella and Enterobacteria, and Candidatus Saccharimonas was strongly negatively correlated with Escherichia–Shigella (Supplementary Figure 2). The enriched Muribaculum in the CLP group was strongly negatively correlated with the enriched Lactobacillus in the GM group and strongly positively associated with the enriched Lachnospiraceae UCG-006 in the CLP group. The enriched Akkermansia in the CLP group was strongly positively correlated with Terrisporobacter, Proteus, and Odoribacter. Candidatus Saccharimonas enriched in the GM group was strongly negatively correlated with Escherichia–Shigella enriched in the CLP group (Supplementary Figure 3).

**Functional alterations of GM on gut microbiota in the CLP group**

To characterize the functional alterations of gut microbiota following GM application in rats with sepsis, we used Tax4Fun to analyze 16S rRNA and predicted functional composition profiles. Eighteen pathways were differentially enriched ($P < 0.05$) between the CLP and GM groups (Supplementary Figure 4). Then, we investigated the correlation between different species ($n = 27$) and KEGG pathways ($n = 44$) using Spearman correlation analysis (Student t-test, Spearman correlation coefficient $<-0.80$ or $>0.80$, $P < 0.05$) (Supplementary Figure 5). CLP-enriched Akkermansia was negatively correlated with phospholipase D signaling pathway and carbohydrate metabolism signaling pathway. GM-enriched Lactobacillus is associated with several pathways, such as cell cycle, tight junction, phosphotransferase system, isoflavone biosynthesis, caffeine metabolism, thyroid hormone synthesis, mineral absorption, and cellular motility and secretion, and positively correlated with transport and cancer pathways. GM-enriched Ruminococcus was positively correlated with amino sugar and nucleotide sugar metabolism, while Lactobacillus was negatively associated with apoptosis, lipopolysaccharide biosynthesis, steroid hormone biosynthesis, transport, sphingolipid metabolism, and pathways in cancer, fluid shear stress, and atherosclerosis. The dominant Phascolarctobacterium in the CLP group was positively correlated with G protein coupled receptors. Moreover, the dominant Colidextribacter in the CLP group was positively
correlated with polyketide sugar unit biosynthesis, terpomycin biosynthesis, and N glycan biosynthesis (Supplementary Figure 6).

Metabolite analysis of the SC, CLP, and GM groups

Six samples and a QC sample were run during the running process. The aggregation of the QC samples was good, indicating that the method was stable and experimental data had high quality (Supplementary Figure 7). The total ion chromatograms of the SC, CLP, and GM groups were observed (Supplementary Figure 8). In PCA, the SC group was closer to the GM group and was significantly separated from the CLP group (Figures 3A and 3D). Using OPLS-DA, the SC group and CLP group and CLP group and GM group can be divided according to their metabolic differences, and the distinction is significant (Figures 3B-C, 3E-F).

Differential metabolite identification

Multivariate statistical analysis was used to screen differential metabolites (VIP > 1.0 and P < 0.05). In the positive ion mode, the SC group had 28 differential metabolites compared with the CLP group, eight were significantly upregulated in the SC group, and 20 were significantly downregulated in the SC group (Figure 4A). The GM group had 26 differential metabolites compared with the CLP group: 13 were significantly upregulated and 13 were significantly downregulated (Figure 4B). In the negative ion mode, the SC group had 17 differential metabolites compared with the CLP group: 12 were significantly upregulated and 5 were significantly downregulated (Figure 4C). The GM group had 20 differential metabolites compared with the CLP group: 16 were significantly upregulated and 4 were significantly downregulated (Figure 4D). There were 15 significantly changed metabolites in the SC, CLP, and GM groups. The detailed information of each metabolite is shown in Table 2. As shown in Figure 4E, 15 metabolites are mainly divided into 11 fatty acids and 4 other classified metabolites, namely, 10E, 12Z-octadecadienoic acid, 9,10-epoxyoctadecenoic acid, 15-KETE, and alpha-linolenic acid. These four fatty acids are grouped together, and the relative abundance is lower in the CLP group but higher in SC and GM groups.

Metabolic pathway analysis of key differential metabolites

Using MetaboAnalyst 3.0 and the KEGG database, the different metabolite pathways between the CLP and GM groups were analyzed. It was found that GM mainly regulates sphingolipid metabolism, histidine metabolism, steroid biosynthesis, glycerophospholipid metabolism, primary bile acid biosynthesis, and alpha-linolenic acid metabolism (Supplementary Figure 9). They are consistent with the predicted pathway of Tax4Fun function, and the GM group was negatively related to apoptosis, lipopolysaccharide biosynthesis, steroid hormone biosynthesis, and sphingolipid metabolism. Therefore, changes in gut metabolites after GM application may be related to gut microbiota dysbiosis.

Links between different genera and metabolites

To further investigate the microbiota–intestinal metabolite relationship after GM application, Spearman's correlation analysis was used to determine the connections between 27 different intestinal genera and
differential metabolites between the CLP and GM groups. Moreover, we used a heatmap to illustrate their correlations (Spearman's correlation coefficient \(< -0.80\) or \(> 0.80, P < 0.05\)). Some CLP-rich genera (e.g., Akkermansia) are positively associated with chenodeoxycholic acid 3-sulfate, cholesterol, lysophosphatidylethanolamine (LysoPE) (16:1(9Z)/0:0), LysoPE (16:0/0:0), LysoPE (15:0/0:0), and PC-M6 and negatively correlated with 15-KETE, sphinganine, and Choline. CLP-rich genera (e.g., Escherichia–Shigella) was positively correlated with chenodeoxycholic acid 3-sulfate, calystegnin A3, and [10]-gingerdione and negatively correlated with 15-KETE. CLP-rich genera (e.g., Proteus) was positively correlated with chenodeoxycholic acid 3-sulfate, cholesterol, LysoPE (16:0/0:0), LysoPE (15:0/0:0), and [10]-gingerdione and negatively correlated with 15-KETE. Moreover, the GM-rich genus (Lactobacillus) was negatively correlated with LysoPE (15:0/0:0). Among them, LysoPE (15:0/0:0) was positively correlated with CLP-rich genera (Akkermansia and Proteus) and had a negative correlation with GM-rich genera (Lactobacillus) (Figure 5A).

As shown in Figure 5B, some CLP-rich genera (e.g., Akkermansia) were positively correlated with quinolinic acid and negatively correlated with leukotriene B4, 9,10-DHOME, deoxycholic acid, L-cysteine, phenylacetylglycine, N-acetylvaline, and alpha-linolenic acid. Escherichia–Shigella enriched in the CLP group was positively correlated with quinolinic acid and 5a-tetrahydrocorticosterone and negatively correlated with leukotriene B4, 9,10-DHOME, and m-coumaric acid. CLP-rich genera (Proteus) were positively correlated with quinolinic acid, 5a-tetrahydrocorticosterone, and cortisol and negatively correlated with leukotriene B4, 9,10-DHOME, deoxycholic acid, N-alpha-acetylsine, oleoyl glycine, m-coumaric acid, and alpha-linolenic acid. Furthermore, GM-rich genus (Lactobacillus) was positively correlated with leukotriene B4, 9,10-DHOME, N-alpha-acetylsine, N-acetylvaline, phenylacetylglycine, and oleoyl glycine and negatively correlated with quinolinic acid. Among them, leukotriene B4 and 9,10-DHOME were negatively correlated with CLP-rich genera (Akkermansia, Escherichia–Shigella, and Proteus) and positively correlated with the GM-rich genus (Lactobacillus). N-Alpha-acetylsine and oleoyl glycine were negatively correlated with the CLP-rich genus (Proteus) and positively correlated with the GM-rich genus (Lactobacillus). N-Acetylvaline and phenylacetylglycine were negatively correlated with CLP-rich Akkermansia and positively correlated with GM-rich Lactobacillus. Quinolinic acid was positively correlated with CLP-rich genera (Akkermansia, Escherichia–Shigella, and Proteus) and negatively correlated with GM-rich genera (Lactobacillus).

Effects of GM on lung damage and survival in rats with sepsis

The mortality rate in the CLP group (10/20) at 24 h after operation was higher than in the SC group (0/20) \((P = 9.60335E-05)\) and GM group (4/20) \((P = 0.048)\). Inflammatory infiltration, edema, and structural damage of the lung tissue in the CLP group were significantly worse than those in SC and GM groups (Figure 6).

Discussion

In this study, we found the beneficial effects of GM on intestinal microbiota and metabolic profile in rats with sepsis. The microbial composition of CLP rats was significantly altered, such as the predominance of
Proteobacteria, Akkermansia, and Escherichia–Shigella and reduction of Lactobacillus. However, the GM group showed a reverse in the dysbiosis of the gut microbiota, and the composition was similar to that in the SC group. The GM changed 26 and 20 differential metabolites in the positive and negative ion modes, respectively, by regulating sphingolipid metabolism, histidine metabolism, steroid biosynthesis, glycerophospholipid metabolism, primary bile acid biosynthesis, and alpha-linolenic acid metabolism. GM-regulated microorganisms, such as Akkermansia, Escherichia–Shigella, Proteus, and Lactobacillus, were associated with differential metabolites (LysoPE (15:0/0:0), quinolinic acid, leukotriene B4, and 9,10-DHOME). Moreover, GM could also reduce the lung tissue damage caused by sepsis and improve the survival rate of rats with sepsis.

The gut microbiota could contribute to host physiology by producing numerous metabolites that act as signaling molecules and substrates for metabolic responses[34]. Fecal-based metabolomics can comprehensively reflect metabolic profiles, and the combination of gut microbiota and metabolomics can reflect the interactions between them. Lactobacillus as a natural gastrointestinal flora have various beneficial effects, including immune modulation, interference with intestinal pathogens, and maintenance of healthy intestinal flora that are widely used in the prevention of Clostridium difficile infection[35]. Lactobacillus are increasingly important in maintaining gut barrier function and regulating mucosal and systemic immune responses[36]. The use of Lactobacillus significantly reduced the risk of late-onset sepsis[37]. Lactobacillus administration at the onset of sepsis may improve sepsis survival by improving intestinal epithelial homeostasis and intestinal permeability and reducing local and systemic inflammatory responses[38]. Lactobacillus could also reduce sepsis-induced liver damage[39], and reverse sepsis-induced microbiota dysbiosis[40]. Preventive use of Lactobacillus-containing synbiotics could improve intestinal dysbiosis and prevent enteritis and ventilator-associated pneumonia in patients with sepsis[41].

Although Akkermansia has an anti-obesity effect[42, 43], its abundance could also disrupt host mucins, induce intestinal inflammation and promote colon tumorigenesis[44, 45]. Increased abundance of Akkermansia and decreased short-chain fatty acids -producing bacteria (Muribaculaceae, Lachnospiraceae, Lachnospiraceae_NK4A136_group, Roseburia) aggravate colorectal cancer[46]. Seibert et al. found that Akkermansia abundance was significantly increased at peak viral infection and allergic diseases[47, 48]. Akkermansia could increase intestinal permeability and expose the enteric plexus to oxidative stress[49]. Our previous study found an increased proportion of Escherichia shigella associated with lipopolysaccharide production in rats with sepsis[50]. Escherichia–Shigella induces macrophage death that spreads to surrounding epithelial cells, and activates host immune responses[51].

LysoPE is the product of hydrolysis phosphatidylethanolamine (PE) by phospholipase A2 and a component of cell membranes[52]. LysoPE could stimulate natural killer T cell activation via autoantigenicity and may play a role in innate immunity during infection[53]. Lipopolysaccharide administration increased endogenous phospholipase A activity by 73.5% and LysoPE content by 1.4-fold[54]. Plasma phospholipase A2 levels are higher in patients with sepsis and significantly correlated with
TNF-α, IL-6, and IL-8 levels[55]. LysoPE(16:0/0:0) levels were increased in rats with sepsis-induced liver injury due to extensive cell membrane disruption[56, 57].

9, 10-DHOME is a derivative of linoleic acid and ligand for peroxisome proliferator-activated receptor gamma 2 (PPAR-γ2)[58]. PPAR-γ is a lipid-activated transcription factor that regulates genes related to lipid metabolism[59] and the differentiation of monocytes, T cells, and NK cells[60]. Metabolite downstream of indoleamine 2,3-dioxygenase (IDO), such as quinolinic acid is increased in sepsis[61]. Moreover, antibiotic-mediated release of TNF-α may activate IDO to produce neurotoxic quinolinic acid[62]. The tryptophan metabolite–quinolinic acid synthesized in activated macrophages acts as an excitatory transmitter at N-methyl-D-aspartate (NMDA) receptors. Studies showed that activated NMDA receptors activate nitric oxide synthase, release free radicals, damage DNA and activate poly-ADP-ribose-synthetase, resulting in energy consumption and cell death. These were severe main causes of metabolic encephalopathy in patients[63]. Plasma quinolinic acid levels showed good discrimination between patients with and without sepsis, with an area under the curve of up to 0.832[64].

Our study is the first to elucidate the relationship between intestinal microbiota and metabolomics after GM administration in sepsis. GM could affect the relative abundance of metabolites by changing the gut microbiota composition. It reveals the potential mechanism that GM could be used to treat lung damage and improve prognosis in rats with sepsis. However, the study also has some limitations. First, the experiments had a small sample size, and the research results need to be verified with more animal experiments and clinical studies. Furthermore, although the effect of GM on lung damage using HE-stained sections was observed, a more complete experimental design is needed to explore the molecular mechanism by which GM improves sepsis prognosis.

**Conclusion**

After GM administration, the relative abundance of Escherichia–Shigella and Akkermansia in rats with sepsis decreased, and that of Lactobacillus was increased. Moreover, the differential metabolites were mainly enriched in sphingolipid metabolism, histidine metabolism, steroid biosynthesis, glycerophospholipid metabolism, primary bile acid biosynthesis, and alpha-linolenic acid metabolism. Combined analysis found strong correlations between key bacterial genera and differential metabolites on metabolic pathways. GM could affect the changes of Lactobacillus, Akkermansia, Escherichia–Shigella and other genera and regulate the relative abundance of LysoPE (15:0/0:0), quinolinic acid, leukotriene B4, and 9,10-DHOME that attenuate sepsis-induced lung injury, which provides a potential mechanism for the application of GM in sepsis therapy.

**Declarations**

**Author Contributions**
All the authors contributed substantially to the work presented in this article. TWS and conceived of the study. YQC, XJZ and YZW contributed to the initial draft. ZHZ, XFD, HYL, DW and YLS contributed to the study protocol. SHL, XGD and YBL contributed to the visualization of experimental results. TWS revised the article. All authors have approved the final and submitted version of the manuscript.

**Availability of Data and Materials**

The datasets used and/or analysed in the present study are available from the corresponding author on reasonable request.

**Ethical Approval and Consent to Participate**

All animal experiments were conducted according to the guidelines established by the National Institutes of Health (http://grants1.nih.gov/grants/olaw/) and approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Approval No. 2019-KY-235).

**Acknowledgments**

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**Disclosures**

Yuqing Cui, Xiaojuan Zhang, Yuze Wang, Zihao Zhang, Xianfei Ding, Huoyan Liang, Dong Wang, Yali Sun, Shaohua Liu, Xiaoguang Duan, Yibin Lu, Tongwen Sun has nothing to disclose.

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**Conflicts of Interest**

The authors have no conflicts of interest to disclose.

**References**


Tables
Table 1
Diversity analysis of a single sample with different groups under the 97% consistency threshold.

<table>
<thead>
<tr>
<th>Group</th>
<th>Simpson</th>
<th>Shannon</th>
<th>Chao1</th>
<th>ACE</th>
<th>Goods coverage</th>
<th>PD whole tree</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>0.947</td>
<td>5.711</td>
<td>475.762</td>
<td>479.040</td>
<td>&gt;99.8%</td>
<td>33.920</td>
</tr>
<tr>
<td>CLP</td>
<td>0.873</td>
<td>4.911</td>
<td>428.522</td>
<td>436.556</td>
<td>&gt;99.8%</td>
<td>28.936</td>
</tr>
<tr>
<td>GM</td>
<td>0.903</td>
<td>4.993</td>
<td>408.385</td>
<td>413.499</td>
<td>&gt;99.8%</td>
<td>39.722</td>
</tr>
</tbody>
</table>
Table 2
Detailed information of differential metabolites of the SC, CLP, and GM groups (VIP > 1, P < 0.05).

<table>
<thead>
<tr>
<th>Differential metabolites</th>
<th>RT</th>
<th>m/z</th>
<th>VIP</th>
<th>Fold change (CLP/SC)</th>
<th>Fold change (CLP/GM)</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>[10]-Gingerdione</td>
<td>79.03</td>
<td>349.23</td>
<td>1.78</td>
<td>3.40</td>
<td>2.98</td>
<td>Benzenoids</td>
</tr>
<tr>
<td>PC-M6</td>
<td>225.00</td>
<td>422.27</td>
<td>1.58</td>
<td>3.11</td>
<td>2.79</td>
<td>Organoheterocyclic compounds</td>
</tr>
<tr>
<td>Calystegin A3</td>
<td>291.51</td>
<td>160.10</td>
<td>1.73</td>
<td>2.80</td>
<td>1.93</td>
<td>Alkaloids and derivatives</td>
</tr>
<tr>
<td>LysoPE(16:1(9Z)/0:0)</td>
<td>224.04</td>
<td>452.28</td>
<td>1.85</td>
<td>4.90</td>
<td>2.49</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>LysoPE(16:0/0:0)</td>
<td>222.75</td>
<td>454.30</td>
<td>1.25</td>
<td>3.77</td>
<td>0.19</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>5-Hexyltetrahydro-2-furanoctanoic acid</td>
<td>224.98</td>
<td>299.26</td>
<td>1.58</td>
<td>3.08</td>
<td>2.82</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>25.57</td>
<td>369.35</td>
<td>1.39</td>
<td>2.08</td>
<td>1.72</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>LysoPE(15:0/0:0)</td>
<td>224.99</td>
<td>440.28</td>
<td>1.61</td>
<td>3.11</td>
<td>2.89</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>15-KETE</td>
<td>254.16</td>
<td>336.25</td>
<td>1.42</td>
<td>0.33</td>
<td>0.36</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>Chenodeoxycholic acid 3-sulfate</td>
<td>281.50</td>
<td>473.26</td>
<td>1.83</td>
<td>6.31</td>
<td>17.54</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>Ethyl beta-D-glucopyranoside</td>
<td>292.10</td>
<td>209.10</td>
<td>1.81</td>
<td>4.71</td>
<td>3.19</td>
<td>Organic oxygen compounds</td>
</tr>
<tr>
<td>10E,12Z-Octadecadienoic acid</td>
<td>40.63</td>
<td>279.23</td>
<td>1.30</td>
<td>0.46</td>
<td>0.57</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>Alpha-Linolenic acid</td>
<td>43.74</td>
<td>277.22</td>
<td>1.92</td>
<td>0.34</td>
<td>0.33</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>9,10-Epoxoctadecenoic acid</td>
<td>48.03</td>
<td>295.23</td>
<td>1.37</td>
<td>0.48</td>
<td>0.41</td>
<td>Lipids and lipid-like molecules</td>
</tr>
<tr>
<td>5a-Tetrahydrocorticosterone</td>
<td>146.75</td>
<td>349.24</td>
<td>1.05</td>
<td>2.44</td>
<td>-</td>
<td>Lipids and lipid-like molecules</td>
</tr>
</tbody>
</table>

Figures
Figure 1

Effect of GM on the gut microbiota of rats with sepsis. A. Rarefaction curves plotted for each sample. B. The Venn diagram of the OTUs of each group. C. Histogram of relative abundance of top ten species at the phylum level. D. The ratio of Firmicutes to Proteobacteria in the SC, CLP, and GM groups; *compared with the CLP group, *P < 0.05. E. Relative abundance of the top ten species at the genus level. F. PcoA analysis based on weighted Unifrac distance.

Figure 2
Comparison of abundances of biomarkers with statistical differences in different groups. A. Linear discriminant analysis (LDA) relative abundance map of differential bacterial genera between the CLP and SC groups. B. Linear discriminant analysis (LDA) relative abundance map of differential bacterial genera between the CLP and GM groups. C. Linear discriminant analysis (LDA) relative abundance map of differential bacterial genera between the SC and GM groups.

**Figure 3**

A–C represent positive ion mode; D–F represent negative ion mode. A and D represent the PCA diagrams of the SC, CLP and GM groups. B and E represent the OPLS-DA diagrams of the SC and CLP groups. C and F represent the OPLS-DA diagrams of the GM and CLP groups.
Figure 4

Heatmap of fecal differential metabolites in positive and negative ion mode. Red indicates highly expressed, and blue indicates expressed at a low level. A. SC and CLP group (positive ion mode). B. GM group and CLP group (positive ion mode). C. SC and CLP group (negative ion mode). D. GM group and CLP group (negative ion mode). E. Heatmap of common differential metabolites of the three groups.

Figure 5

Heatmap between different genera of intestinal microbiota and metabolites in CLP and GM groups. Red, positive correlation; blue, negative correlation. × mean $P < 0.05$. A. Positive ion mode. B. Negative ion mode.
Figure 6

A–C. Histopathological section of the lung tissue in the SC, CLP, and GM groups, respectively (bar = 100 μm).

Supplementary Files

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- SupplementaryFigure9.tif