RESEARCH

Gut microbiota disruption during sepsis and the influence of innate metabolites on sepsis prognosis

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Abstract

Sepsis causes high mortality in intensive care units. Although there have been many studies on the gut microbiota in patients with sepsis, the impact of sepsis on the gut microbiota has not been directly determined because the treatment of sepsis also affects the gut microbiota. Therefore, we designed this animal experiment to explore gut microbiota alterations during sepsis. Mice were divided into two groups, mice that survived less than 3 days and mice that survived more than 3 days. Fecal samples collected on the day of cecal ligation and puncture (CLP), as well as on the 3rd and 7th days after CLP, were subjected to microbial community analysis and nontargeted metabolomics analysis. The results showed significantly lower bacterial diversity in fecal samples after CLP. At the genus level, the fecal samples obtained on the 3rd and 7th days after CLP exhibited significantly increased relative abundances of *Bacteroides*, *Helicobacter*, etc., and significantly decreased relative abundances of *Alloprevotella*, *Prevotella*, etc. Innate metabolite levels were significantly different in mice that survived less than 3 days and mice that survived more than 3 days. In conclusion, CLP-induced sepsis in mice changes the structure of the gut microbiome, and innate metabolites affect the prognosis of septic mice.

Keywords Sepsis · Microbiota · Microbiome · Metabolite · *Alloprevotella* · *Bacteroides*

Introduction

Sepsis is a life-threatening type of organ dysfunction caused by a dysregulated host response to an infection (Singer et al. 2016). Sepsis affects one-fifth of patients admitted to intensive care units (ICUs) in mainland China, with a 90-day mortality rate of 35.5% (Xie et al. 2020).

The gut microbiota is a complex ecosystem that includes a wide variety of bacteria, consisting of trillions of bacterial cells (Sender et al. 2016). Many studies have shown that these microbial communities are involved in many physiological activities, such as metabolism, control of the nervous system (Adak and Khan 2019), and regulation of the immune response (Rooks and Garrett 2016). In addition to the gut microbiota, the difference of metabolites caused by diets and lifestyles will also affect the immune status, leading to different outcomes of diseases (Leitner et al. 2023; Perler et al. 2023).

Although the pathogenesis of sepsis is multifactorial and not fully understood, an increasing amount of evidence shows that disruption of the gut microbiota predisposes patients to sepsis and negatively affects sepsis outcomes (Adelman et al. 2020). Changes in the gut microbiota do not only affect outcomes before the onset of sepsis; after the onset of sepsis, changes in the structure of the normal gut microbiota worsen and lead to worse outcomes.

There are many studies on the gut microbiota in patients with sepsis, but they have not directly determined the influence of sepsis, which is a single variable, on the gut microbiota because the gut microbiota is affected by sepsis treatments, such as antibiotics, opioids, parenteral nutrition, and proton pump inhibitors (Zaborin et al. 2014; Imhann et al. 2016; Dahlgren et al. 2019). Therefore, we designed this animal experiment. We found that 3 days after the operation was the peak time of death for septic mice. Therefore, we divided the mice into groups according to the postoperative survival time and explored the changes in the gut microbiota after sepsis and the effect of innate metabolites on the prognosis of mice.

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Materials and methods

Animals

SPF-grade, 6- to 8-week-old male C57BL/6 J mice were purchased from Charles River. All animals were housed in a specific pathogen-free facility and given free access to food and water. The animal studies were approved by the Harbin Medical University Animal Care and Use Committee.

Experimental design

The mice were subjected to cecal ligation and puncture (CLP) surgery. The survival of the mice was recorded every day after the operation, and a survival curve was drawn (Suppl. Fig. 1). According to the postoperative survival time, the CLP mice were divided into two groups: mice that survived less than 3 days and mice that survived more than 3 days (8 mice in each group). We collected the feces from mice that survived more than 3 days on the day of surgery (preoperative), the 3rd day after surgery, and the 7th day after surgery, and the faces from the mice that survived less than 3 days on the day of surgery. Feces were collected in a sterile cryopreservation tube at 3 p.m. every day and stored in a -80 °C freezer after collection. Subsequently, the feces were used for microbial community analysis and untargeted metabolomics analysis.

CLP model

The mice were subjected to CLP, an established model to induce polymicrobial peritonitis. Mice were anesthetized before surgery with an animal anesthesia ventilator system. After opening the abdomen, the cecum was exteriorized and ligated 1 cm from the terminus of the cecum without obstructing intestinal continuity. The cecum was punctured once with a 23-G needle and squeezed to excrete a small amount of fecal material. The abdominal wall was closed in layers after placing the cecum back in the abdomen. After surgery, all animals were given a subcutaneous injection of 1 ml of physiological saline that had been prewarmed to 37 °C. To evaluate mortality, the mice were observed for 7 days after surgery. The animals were sacrificed 7 days after surgery.

Fecal collection

To ensure the sterility of the fecal collection environment as much as possible, a plastic box used exclusively for fecal collection was sterilized and desiccated, and the mice were placed in the plastic box. After the mice defecated, the feces were immediately collected into sterile cryopreservation tubes and placed in liquid nitrogen for quick freezing. The samples were then immediately placed at -80 °C.

Microbial community analyses

Microbial DNA was extracted from the fecal samples using the QIAamp® Fast DNA Stool Mini Kit according to the manufacturer's protocols. The V3-V4 region of the bacterial 16S ribosomal RNA genes was amplified by PCR using primers 341F 5'-CCTACGGGRSGCAGCAG)-3' and 806R5'-GGACTA CVVGGGTATCTAATC-3'. Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using Qubit® 2.0 (Invitrogen, U.S.). After preparation of the library, the tags were sequenced on a MiSeq platform (Illumina, Inc., CA, USA) for paired-end reads of 250 bp, which overlapped on their 3 ends for concatenation into original longer tags.

Untargeted metabolomics GC−MS analyses

The feces were collected in 2-mL EP tubes containing 500 μL of prechilled solution and 10 μL of ribitol, and then, the mixtures were ground and centrifuged for 15 min (12,000 rpm, 4 °C). A total of 400 μL of supernatant was collected, and 80 μL of each sample was mixed for quality control (QC) samples. The supernatant was dried in a vacuum centrifuge. After the addition of 30 μL methoxyamine salt reagent to the dried metabolites, the samples were incubated at 80 °C for 30 min. Then, 40 μL of BSTFA (containing 1% TMCS, v/v) was added to each sample and incubated at 70 °C for 1.5 h. After cooling to room temperature, 5 μL of FAMEs were added to the sample. Subsequently, an Agilent 7890 gas chromatography-time-of-flight mass spectrometer equipped with an Agilent DB-5MS capillary column (30 m × 250 μm × 0.25 μm, J&W Scientific, Folsom, CA, USA) was used for detection.

Statistical analyses

Operational taxonomic units (OTUs) were clustered with 97% similarity using UPARSE (http://drive5.com/uparse/), and chimeric sequences were identified and removed using Userach (version 7.0). Each representative tag was assigned to a taxon by RDP Classifier (http://rdp.cme.msu.edu/) according to the RDP database (http://rdp.cme.msu.edu/), using a confidence threshold of 0.8. An OTU profiling table was generated, and alpha/beta diversity analyses were performed with Python scripts in QIIME. ChromaTOF software (V 4.3x, LECO) was used to analyze the mass spectrum data, including analysis of peak extraction, baseline correction, deconvolution, peak integration, and peak alignment. In the qualitative analysis of the substances, the LECO-Fiehn Rtx5 database was used, including mass spectrometry matching and retention time index matching.

The data are presented as the means ± SEMs, and GraphPad Prism 7 was used for the statistical analyses. Data sets with 2 groups were analyzed using an unpaired t test. Data sets with more than 2 groups were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Survival was analyzed using the log-rank (Mantel−Cox) test. A p value of < 0.05 indicated statistical significance for all statistical analyses.

Data availability

The data sets generated during the current study have been deposited at NCBI repository under the accession number PRJNA758244.

Results

Alterations in the gut microbiota of septic mice

Principal coordinate analysis (PCoA) unweighted Adonis analysis revealed no significant differences in preoperative fecal samples between mice that survived less than 3 days and mice that survived more than 3 days (Fig. 1A), whereas the gut microbiota of the preoperative fecal samples and the postoperative fecal samples collected on the 3rd and 7th day from mice that survived more than 3 days were significantly separated ($P < 0.001$) (Fig. 1B). Principal component analysis (PCA) also showed that there was no significant difference in preoperative feces between
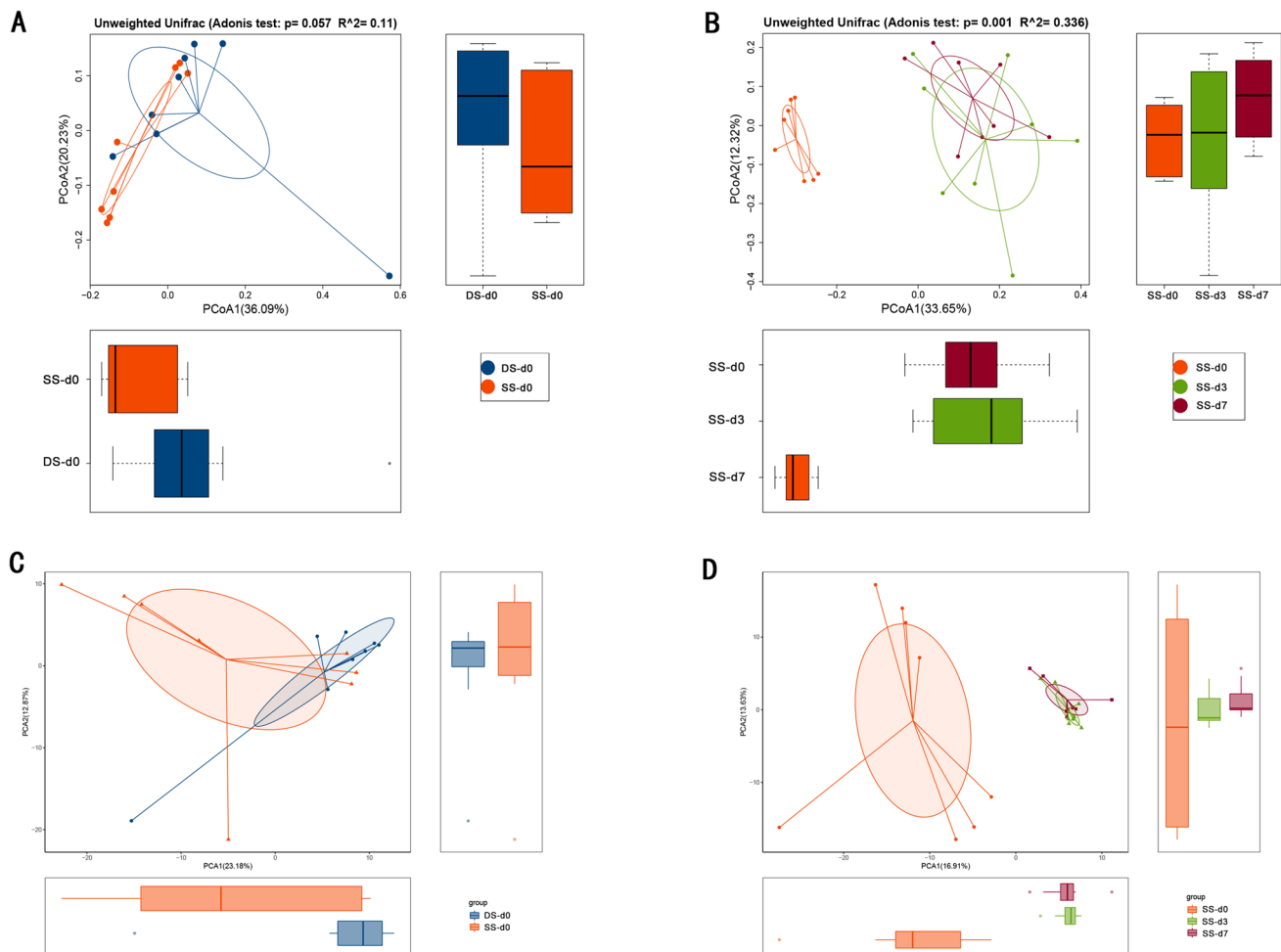
Fig. 1 OTU PCA and PCoA unweighted analysis of changes in the gut microbiota. SS (Survival Sepsis group) indicates that the mice survived less than 3 days; DS (Dead Sepsis group) indicates that the mice survived more than 3 days. (**A**) The PCoA analysis showed that the points between the two groups were aggregative, which means there were no significant differences in the gut microbiota between the two groups. (**B**) The gut microbiota was dispersed from point to point between preoperative and postoperative fecal samples from mice that survived more than 3 days. (**C**) OTU PCA showed that the points between the two groups were aggregative. (**D**) The points among the 3 groups were significantly separated. (Different colors in the figures represent different groups of samples, and the greater the distance between the two points, the greater the difference in microbial community between the two samples)

the mice that survived less than 3 days and those that survived more than 3 days (Fig. 1C), while there were significant differences between preoperative and postoperative fecal samples for mice that survived more than 3 days (Fig. 1D).

The Shannon index showed no significant difference in the alpha diversity of the microbial community between the feces of mice that survived less than 3 days and those of mice that survived longer than 3 days (Fig. 2A). However, in the feces of mice that survived for more than 3 days, there was a significant decrease in alpha diversity at 3 and 7 days postoperatively compared to the preoperative levels (Fig. 2B).

Effects of sepsis induced by the CLP model on variations in the gut microbiota composition of mouse feces

We selected the top 20 bacterial genera with high relative abundance. At the genus level, the fecal samples obtained on the 3rd day and 7th day after CLP exhibited significantly increased relative abundances of *Bacteroides*, *Helicobacter*, *Clostridium sensu stricto*, *Clostridium XlVa*, *Mucispirillum*, *Roseburia*, and *Mycoplasma* and significantly decreased relative abundances of *Alloprevotella*, *Prevotella*, *Parabacteroides*, *Parasutterella*, and *Akkermansia* (Fig. 3A). At the phylum level, the relative abundance of *Bacteroidetes* significantly decreased on the 3rd and 7th days after CLP, and the relative abundance of *Proteobacteria* significantly increased (Fig. 3B).

Linear discriminant analysis effect size (LEfSe) analysis was used to analyze the gut microbiota of preoperative fecal samples and postoperative fecal samples on the 3rd and 7th days from mice that survived more than 3 days, and 72 OTUs with significant differences (LAD > 2.0) were found, including 7 at the phylum level, 8 at the class level, 10 at the order level, 18 at the family level, and 29 at the genus level. At the genus level, among the OTUs with significant differences, *Alloprevotella* and *Prevotella* were the two bacteria with the greatest influence on the difference in the preoperative fecal samples of mice. In the fecal samples on the 3rd day after CLP, *Escherichia/Shigella* and *Clostridium XI* were the top two most influential bacteria. *Clostridium XlVa* and *Enterococcus* were the two bacteria with the largest influence on abundance in the postoperative fecal samples on the 7th day (Fig. 4).

Fig. 2 Alpha-diversity index difference analysis. (**A**) No significant difference in preoperative fecal samples was observed between mice that survived less than 3 days and mice that survived more than 3 days. (**B**) The preoperative fecal samples were significantly different from the samples collected on the 3rd and 7th days. **means $p < 0.01$, ***means $p < 0.001$
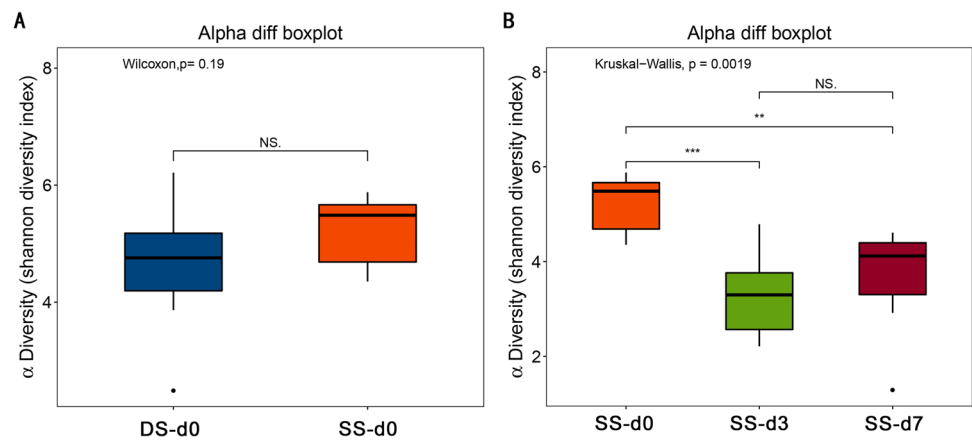
Fig. 3 Species abundance analysis. The abscissa is the group name, and the ordinate is the relative abundance of species. Different colors correspond to different species names, and the length of the color block represents the relative abundance of the species represented by the color block. (**A**) At the genus level. (**B**) At the phylum level
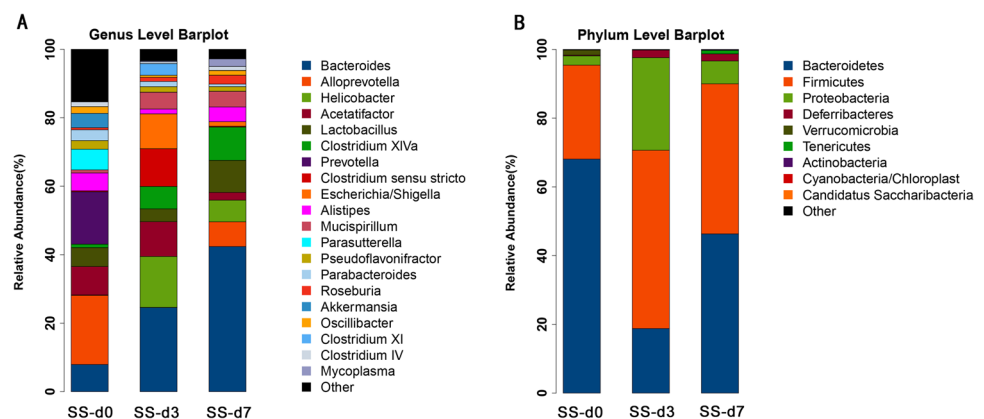
The influence of mouse innate metabolites on the prognosis of sepsis caused by CLP

The preoperative feces of mice that survived less than 3 days and those of mice that survived more than 3 days were analyzed by metabonomics, and we found differences in metabolites between the two groups (Fig. 5A). 3-(3-Hydroxyphenyl)propionic acid, 3-methylamino-1,2-propanediol 2, and D-erythronolactone 2 levels were significantly higher in the preoperative feces of mice that survived less than 3 days; 1-hexadecanol, phenylalanine 1,4-aminobenzoic acid, formononetin, spermidine 2,6-hydroxynicotinic acid, phenylalanine 2, fluorene, 1,2,4-benzenetriol, 2-aminophenol 2,phenyl beta-D-glucopyranoside, 2'-deoxyadenosine, and 2-keto-isovaleric acid 1 levels were significantly higher in the preoperative feces of mice that survived more than 3 days (Fig. 5B). We analyzed the metabolic pathways for metabolic differences and used KEGG, SMPDB, PubChem and other databases to obtain the following metabolic

pathways: beta-alanine metabolism, glutathione metabolism, and arginine and proline metabolism (Fig. 5C).

Correlations between the gut microbiota and gut metabolites

Subsequently, to further investigate the relationships between the gut microbiota and metabolites, we performed a Spearman correlation analysis between 16 metabolites and 18 bacterial taxa (Fig. 6). The results showed that the low abundance of metabolites in the fecal samples of mice that survived more than 3 days, including $D - $erythronolactone $2,3 - $methylamino $- 1,2 - $propanediol $2,3 - (3 - $hydroxyphenyl)propionic acid, was positively correlated with *Escherichia_Shigella* and *Blautia* abundance at the genus level. The high abundance of metabolites in the fecal samples of mice that survived less than 3 days, including 1,2,4-benzenetriol, 4-aminobenzoic acid, phenylalanine 2, etc., was positively correlated with

Fig. 4 LDA EffectSize (LEfSe) difference analysis. Microbial taxa with significant effects in different groups were calculated by using the LDA score obtained via linear regression analysis (LDA)
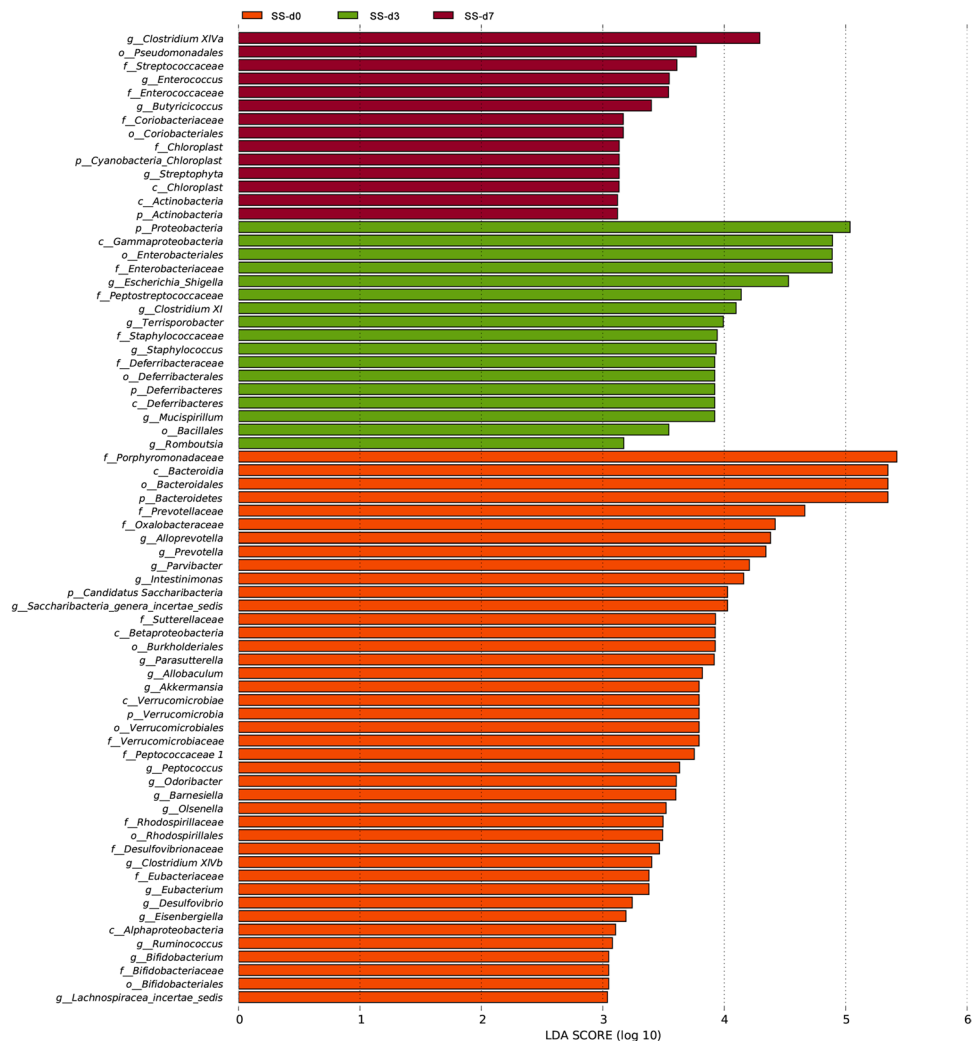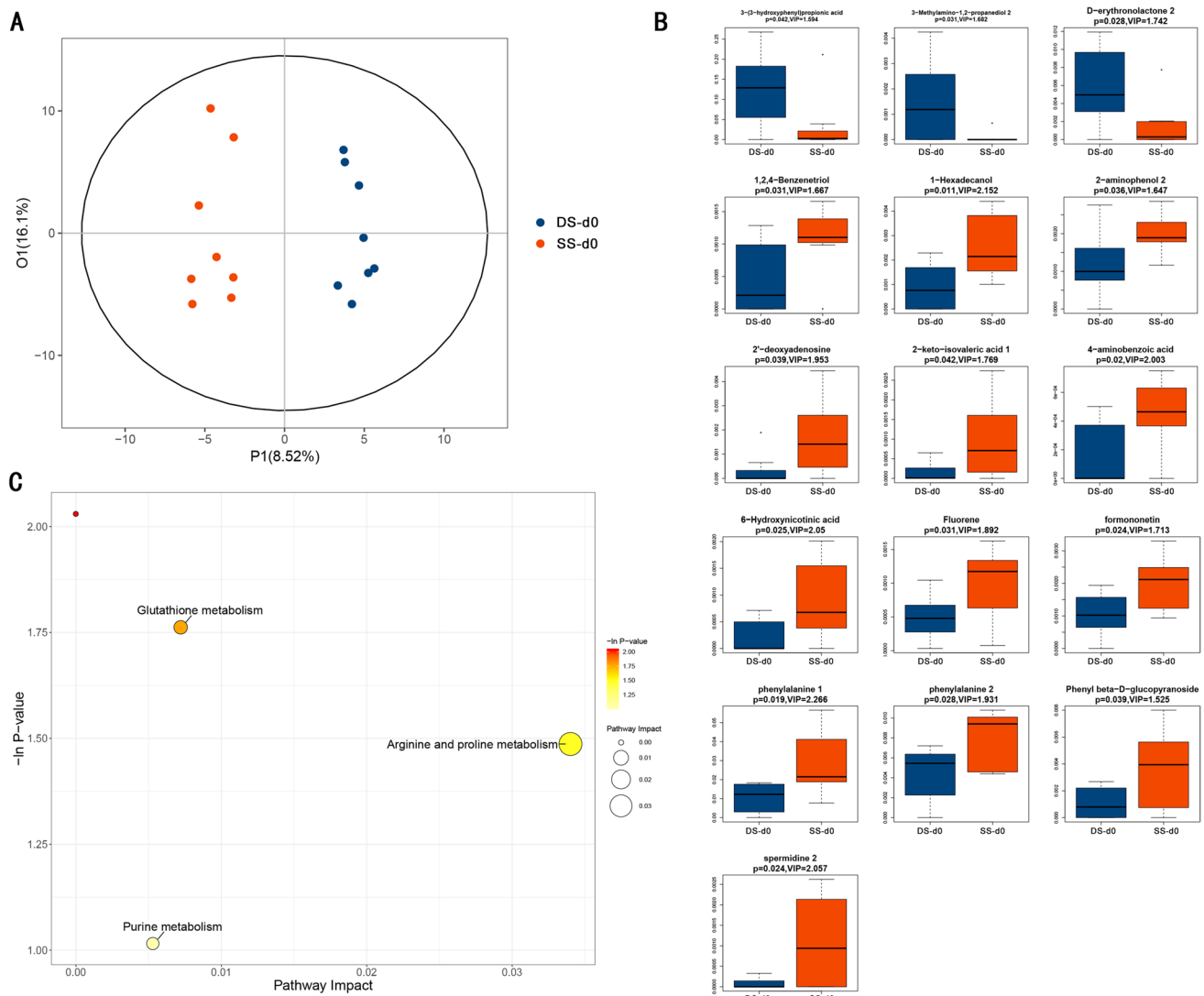
Fig. 5 Difference analysis of metabolites. (**A**) Orthogonal partial least squares-discriminative analysis (OPLS-DA). The abscissa P1 represents the predicted principal component score of the first principal component, and the ordinate O1 represents the orthogonal principal component score. Different colors represent two groups of samples: mice that survived less than 3 days and mice that survived more than 3 days. (**B**) Boxplot of differential metabolites. The abundance of dif-ferent metabolites in the two groups is intuitively displayed in a box-plot. (**C**) Bubble plot of metabolic pathways enriched in differential metabolites. Each bubble in the bubble plot represents a metabolic pathway. The larger bubbles represent a larger influence factor. The deeper the color, the smaller the *p* value, and the more significant the enrichment

Clostridium sensu stricto, *Alloprevotella* and *Desulfovi-brio* abundance at the genus level.

Discussion

First, preoperative fecal samples and postoperative fecal samples collected on the 3rd and 7th days from mice that survived more than 3 days were subjected to 16S rRNA analysis. Research has shown that sepsis can change the gut microbiota. After combining the Adonis analysis of beta diversity and the alpha diversity analysis, we con-cluded that the diversity of the gut microbiome of septic mice was reduced.

Clinical studies have shown that the diversity of the gut microbiome of septic patients is also reduced (Lankelma et al. 2017). In general, the microbial diversity of patients with sepsis declines rapidly on hospital admission, a finding that becomes more pronounced later in their hospitalization (McDonald et al. 2016). In addition, a handful of clinical
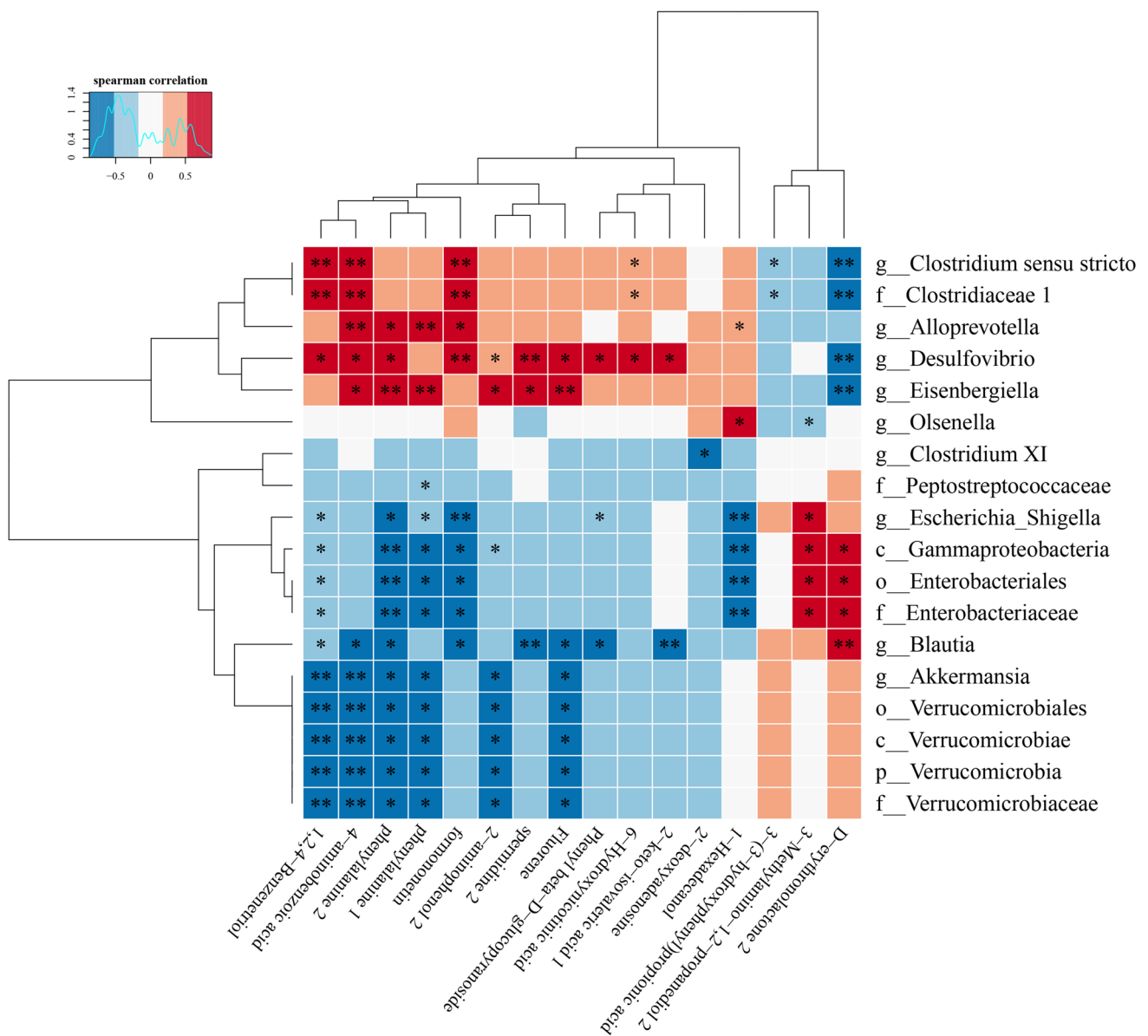
Fig. 6 Correlations between the gut microbiota and gut metabolites. The horizontal coordinate shows the differential metabolites, and the ordinate is the differential species. The color depth indicates the correlation, blue indicates a negative correlation, and red indicates a positive correlation. The p value is the correlation test result; * means $p < = 0.05$, and ** means $p < = 0.01$

studies have shown that low bacterial diversity is associated with poor outcomes. In previous studies, low microbial diversity has been linked to an increased risk of mortality (Shimizu et al. 2011; Taur et al. 2014). This may cause a vicious cycle in which sepsis reduces gut microbial diversity, and lower bacterial diversity aggravates sepsis.

We also found that the composition of the gut microbiome also changed after CLP. Clinical studies by McDonald D et al.(McDonald et al. 2016) and Li Q et al.(Li et al. 2015) confirmed that fecal samples from ICU patients and sepsis patients tend to have decreased *Bacteroidetes* and increased *Proteobacteria* abundance, which corresponds

with our findings. The results of our study showed that *Bacteroidetes* levels in the fecal samples on the 3rd and 7th days were lower than those in postoperative fecal samples at the phylum level, and the decrease was the greatest on the 3rd day after surgery. Studies have shown that *Bacteroides* and *Firmicutes* are crucial producers of short-chain fatty acids (SCFAs), which can regulate gene expression in regulatory T cells and alter the microbiocidal abilities of macrophages (Arpaia et al. 2013; Schulthess et al. 2019). In another study (Hyoju et al. 2019), the microbiota of an experimental group of mice that experienced a combination of pressures, such as an obesogenic Western diet, antibiotic administration and

eventual death from sepsis, was depleted of *Bacteroidetes* and enriched in antibiotic-resistant *Proteobacteria*. This is similar to our mouse microbiota on the 3rd day after CLP. This may explain why the peak time of death of the mice was on the 3rd day after surgery.

At the genus level, compared to that in preoperative fecal samples, an increased relative abundance of *Bacteroides* in the fecal samples was observed on the 3rd day and 7th day after CLP. *Bacteroides* is one of the predominant genera in the human gut and plays an important role in maintaining the stability of the healthy gut ecosystem. Interestingly, according to our hypothesis, these beneficial bacteria should have reduced abundance after sepsis, but this is contrary to our observations. Therefore, we speculated that the increased abundance of this bacterium might have protected the mice from sepsis.

16S rRNA analysis and metabonomics analysis of the fecal samples from the mice that survived less than 3 days and those that survived more than 3 days were performed. There was no significant difference in the composition of the gut microbiota, but the metabolites were quite different. Thus, we suspected that innate metabolites could determine both susceptibility to and severity of infection. The purpose of our experiment is to identify dominant metabolites to resist sepsis. The upregulated metabolites in the feces of mice that survived more than 3 days were considered beneficial to the prognosis of sepsis. Formononetin, an isoflavone extracted from *Astragalus membranaceus*, has exhibited multiple inhibitory effects on cancer (Ong et al. 2019), atherosclerosis (Ma et al. 2020), neuroinflammation (El-Bakoush and Olajide 2018), oxidative stress (Yi et al. 2020) and other diseases in different animal models. Formononetin also protects against bacterial and viral infections (Dai et al. 2019; Wang et al. 2020). Another metabolite, spermidine, is a polycation that is associated with DNA in most living organisms (Pietrocola et al. 2019). Spermidine has been found to be related to a decrease in cancer-related mortality (Pietrocola et al. 2019) and inhibition of oxidative stress and mitochondrial fragmentation (Chai et al. 2019). However, a study by Huang S et al. (Huang et al. 2019) suggested that high phenylalanine levels are associated with a higher mortality risk in cases of severe infection. This is contrary to our conjecture that the highly expressed products phenylalanine 1 and phenylalanine 2 are protective.

We consider that the significantly upregulated metabolites in the feces of the mice that survived less than 3 days are harmful and not conducive to a good prognosis after sepsis. 3-(3-Hydroxyphenyl)propionic acid is produced by the gut microflora through the breakdown of chlorogenic acid and is thereafter absorbed and oxidized in the liver before entering the circulation (Marin et al. 2015). This molecule has recently been characterized as bioactive in stimulating osteoblast activity and increasing bone mass in mice in a dose-dependent manner (Chen et al. 2014).

However, no studies have confirmed that this molecule is related to infection or immunity.

At present, the mechanism of action of intestinal microbial metabolites in the context of sepsis remains unclear. In future experiments, we can supplement protective metabolites before CLP in mice to verify the role and mechanism of these metabolites. If feasible, it can be used for patients with high risk factors of sepsis to improve the outcomes of sepsis, which provides a new idea for the prevention of sepsis.

Compared with clinical studies, animal experiments are not affected by interference effects of treatments, such as antibiotics. Indeed, work by our lab and others has demonstrated that the use of antibiotics can lead to changes in the intestinal microbiota and metabolite levels (Suez et al. 2018; Han et al. 2021). Therefore, our study better reflects the effect of sepsis as a single variable on intestinal flora changes. Moreover, we collected preoperative fecal samples and postoperative fecal samples from mice on the day of surgery, the 3rd and 7th days, which can dynamically reflect the changes in the gut microbiome after sepsis.

In conclusion, CLP-induced sepsis in mice changes the structure of the gut microbiome, and innate metabolites affect the prognosis of septic mice.

Author contributions All authors participated in the design and interpretation of the studies, the analysis of the data and the review of the manuscript. Kaijiang Yu, Changsong Wang and Xiaohui Ma designed the experiments. Xiaohui Ma, Xiaonan Jia, Yahui Peng and Xueting Li conducted the experiments. Xiaohui Ma analyzed the data and wrote the manuscript.

Data availability The datasets generated during the current study are available in the NCBI repository, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA758244.

Declarations

Ethical statement Experiments were performed under a project license (NO. KY2018-02) granted by regional ethics board of Harbin Medical University Cancer Hospital on December 2018, in compliance with chinese guidelines for the care and use of animals. The chairperson of the ethics committee is Changhong Zhao.

Competing interests The authors declare no potential conflicts of interest with respect to the research, authorship, or publication of this article.

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