Int J Clin Exp Pathol 2021;14(6):753-767
www.ijcep.com /ISSN:1936-2625/IJCEP0121908

*Original Article*

# Tracking bacterial DNA patterns in septic progression using 16s rRNA gene amplicon sequencing analysis

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Received September 7, 2020; Accepted February 23, 2021; Epub June 15, 2021; Published June 30, 2021

**Abstract:** Bloodstream infections remain prevalent in intensive care units, leading to a public health challenge worldwide. Routine diagnosis is mainly based on blood culture, but the technique is limited by its time-consuming process and relatively low sensitivity. Emerging molecular diagnostic tools, such as 16S metagenomics, have been developed for detecting bacteria in the blood samples of septic patients. Using a collection of 168 blood samples from 96 septic patients, 16S metagenomics method followed by bioinformatics were applied to study bacterial alterations during the pathogenesis of sepsis. Significant taxonomic variations were found between the two survival groups at different therapeutic time points through sequential 16S metagenomics research. The results on the third day during the treatment course were notably distinct among the studied groups. 16S metagenomics approach can bring novel genetic insight about microbiological fluctuations during septic progression, which may be utilized as a complementary prognostic application. Further etiologic and pathophysiologic explorations are needed to fully explain the linkage between clinical outcomes and genetic changes.

**Keywords:** Bloodstream infection, sepsis, 16S rRNA gene sequencing, disease progression

## Introduction

Pathogenic bacteria can invade into human blood and cause a bloodstream infection, which may result in the transient presence of the pathogens in blood, organ infection, or sepsis. Sepsis may be a fatal condition that refers to a systemic inflammatory response resulting from pathogenic microorganisms intruding into normally sterile tissues, fluids, or body cavities [1]. The causative agent of sepsis can be any microbe, but bacteria account for over 80% of bloodstream infections [2-5]. The most common bacteria isolated from septic sources are Staphylococcus aureus, coagulase-negative Staphylococci, Enterococcus species, Escherichia coli, and Pseudomonas aeruginosa [3]. The morbidity and mortality rates of bloodstream infection are especially high among intensive care units (ICU) and neonatology units in hospitals [3].

Timely diagnosis of sepsis and the correct identification of the pathogen are crucial for formulating antibiotic treatment [6]. Blood culture is the widespread and standard technique for septic diagnosis in most clinical laboratories [7]. However, the conventional blood culture method is time-consuming and not very sensitive. Despite the novel automatization methods in the procedures, the process of the method is still relatively slow (from 6 to 48 h, up to 5 days) [7]. Also, the accuracy and sensitivity of the result could be interfered with by low-concentration and nonculturable bacteria [7]. In this case, molecular technologies for septic diagnosis are attractive alternatives compared to culture-based techniques due to rapid detection and accurate identification of bacterial DNA in blood samples [8].

Next-Generation Sequencing (NGS) is able to identify and sort all species of organisms into their taxonomic classifications. NGS has developed rapidly, and it can read more than a million DNA strands at a time [9]. 16S metagenomic technology can theoretically identify any bacteria in a given sample, which enables the

recognition of unknown pathogens without empirical assumptions. Recently, the technique has been frequently used in studies aimed at analyzing diversity of microbiota in the environment and human gut [10-12]. It is based on the amplification of variable regions in the 16S rDNA with universal primers by PCR and sequencing of the amplicons [13]. For the acquisition of the bacterial identities from the gained sequencing data, bioinformatic approaches are applied where each 16S rDNA amplicon sequence is placed to make a comparison with the reference sequences in public databases. Recently, studies have utilized NGS methods to detect pathogenic bacteria in the blood samples of patients with infectious disease, including sepsis [14-17].

In the effort to investigate the features as well as the variations during disease progression of septic patients from microbiological and etiologic perspectives, we performed NGS-based analysis on plasma samples from 96 patients at different time points in their hospitalization. Bioinformatic trials were implemented to reveal the correlation between changes in the bacterial community within the septic bloodstream and the final clinical outcome.

## Materials and methods

### *Sample collection*

168 blood samples were collected from 96 septic patients who were treated in Beijing Chaoyang Hospital from April 2017 to August 2017. All the included patients were diagnosed with sepsis according to the criteria of the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) [18]. Both Acute Physiology and Chronic Health Evaluation (APACHE) II [19] and Sequential Organ Failure Assessment (SOFA) [1, 20] scoring systems were used to evaluate the severity of the disease.

For all patients included, blood specimens were obtained at sepsis onset (Day 1). Successive sampling after 3-days treatment (Day 3), 5-days treatment (Day 5) and 7-days treatment (Day 7) was conducted. 5-10 ml blood were drawn from peripheral veins of patients and placed in EDTA tubes for subsequent tests. This study was approved by the Ethics Committee of Beijing Chaoyang Hospital. All the patients or their family members were informed and signed a consent for participation.

The obtained specimens were labeled according to the serial numbers of the patients, then grouped on the basis of final clinical outcome. The number format of the samples refers to: survival group ("A" for alive or "B" for dead) + patients' serial number (from "00" to "95") + sampling day ("1", "3", "5" or "7"). The group numbers were the combination of survival state ("A" or "B") + sampling day ("1", "3", "5" or "7").

### *DNA extraction and library construction*

Whole blood samples were centrifuged at 1600 g for 10 min at 4°C within 8 h of collection, then the obtained plasma samples were stored at -80°C before DNA preparation and sequencing.

300 μL plasma was used for DNA extraction through the PowerSoil DNA Isolation Kit (TIANGEN BIOTECH, Beijing, China).

The V3-V4 region of the bacterial 16S rRNA gene was amplified with the reported universal primers [13], which fused to the overhang of Illumina adapter sequences. The library preparation process was followed by the 16S Metagenomic Sequencing Library Preparation Protocol-Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System (Illumina, San Diego, CA). After quality inspection, next generation sequencing was performed using Illumina HiSeq 2500. The effective tags for further analysis were obtained through PE reads assembled through FLASH v1.2.7 [21], purification of raw tags using Trimmomatic v0.33 [22], and elimination of unqualified sequences by UCHIME v4.2 [23]. Singletons, chimeras, contamination, and human sequences were removed during the data filtering steps.

### *NGS data processing*

Using QIIME https://developer-platform.biocloud.net/-ref1 software, reads were clustered through UCLUST and were sorted into operational taxonomic unit (OTU) and annotated based on SILVA (for bacteria, http://www.arb-silva.de) [24] and UNOTE (for fungus, http://unite.ut.ee/index.php) [25] reference databases. OTUs clustering at a threshold of 97% se-

**Table 1.** Admission profile of septic patients and counts of samples in each group

| Survival Group | | A (alive) | B (dead) | Total |
|---|---|---|---|---|
| Information of patients | | | | |
| | | A (n=72) | B (n=24) | Total (n=96) |
| Age (Mean) | | 71 | 72 | 72 |
| Gender | Male | 49 | 12 | 61 |
| | Female | 23 | 12 | 35 |
| Infection Site | Lung | 63 | 9 | 72 |
| | Other | 19 | 3 | 22 |
| APACHE II Score[a] (Average) | | 22 | 28 | 23 |
| Admitting SOFA[b] (Average) | | 9 | 14 | 11 |
| Number of samples | | | | |
| | | A (n=131) | B (n=37) | Total (n=168) |
| Sampling day | Day 1 | 72 | 24 | 96 |
| | Day 3 | 33 | 11 | 44 |
| | Day 5 | 18 | 2 | 20 |
| | Day 7 | 8 | 0 | 8 |

[a]Acute Physiology and Chronic Health Evaluation [19]. APACHE II is based upon 12 initial routine physiologic measurements, age, and previous health status to measure the severity of disease. [b]Sequential Organ Failure Assessment [20]. The SOFA score comprises scores from six organ systems, graded from 0 to 4 according to the degree of dysfunction.

quence similarity were obtained using USEARCH version 10.0 [26]. Taxonomic identification was assigned using the Ribosomal Database Project classifier version 2.2 (http://sourceforge.net/projects/rdpclassifier/) [27]. The noRoot OTU was removed as it represented non-bacterial DNA amplification due to the cross-reactivity of 16S primer to human DNA. Relative OTU abundances and phylogenetic distance metrics were calculated using QIIME for Alpha Diversity analysis and further plotted as Rarefaction Curves [28]. Bray-Curtis algorithm was applied for the calculation of Beta Diversity analysis, and the results were graphed into Analysis of Similarities (Anosim), Principal Coordinates Analysis (PCoA) and Heatmap plots. The Line Discriminant Analysis Effect Size (LEfSe) analysis, was implemented to find the representative biomarkers on the basis of linear discriminant analysis (LDA) where the significant threshold of logarithmic LDA score was set at 4.0. All the results were visualized using QIIME https://developer-platform.biocloud.net/-ref1 software and R language [29].

## Results

*Sample characterization*

61 male and 35 female patients were included in the study. The mean age of the patients was 72 years old. In terms of the mortality, 25% (24 of 96) of the patients died after their admission. The average APACHE II score evaluating disease severity was 23 [19], while the average admitting SOFA score assessing organ failure was 11 [20]. The dominant source of infection was pulmonary (n=72, 75%) (**Table 1**).

*Sequencing quality evaluation*

NGS sequencing obtained an average number of 63,541 raw tags, whereas 58,013 average effective tags were acquired after filtering out the short reads (length ≤35 bp) and unqualified reads.

For evaluating the sequencing adequacy as well as the microbial richness among tested samples, Alpha Diversity Analysis (Rarefaction Curves) were applied to reflect the relativity between the number of sequences and the OTU numbers of each group (**Figure 1**). The result demonstrated distinctions between different sampling points, which showed a significant increase in the OTU numbers in both Day 3 groups.

*Bioinformatic characterization*

The calculation of relative taxonomic abundance showed the taxonomic numbers of the samples at different levels. In brief, the average number of each classification level was 2 (Kingdom), 69 (Phylum), 195 (Class), 333 (Order), 603 (Family), 1391 (Genus) and 1029 (Species), respectively.

Taxonomy distribution analysis was used to exhibit and contrast the overall microbial structure between groups. In the sampled septic population, the Proteobacteria phylum occupied the largest fragment among both A (survival) and B (dead) groups, where the ratio appeared to be over 1/5 (27% and 22%, respectively). This was followed by the phyla of Firmicutes, Cyanobacteria, and Bacteroidetes, the proportions of which were around 20% in the two groups (**Table 2**; **Figure 2**). The distribution histograms were also created at the level of genus to survey the detailed microbiologic traits.

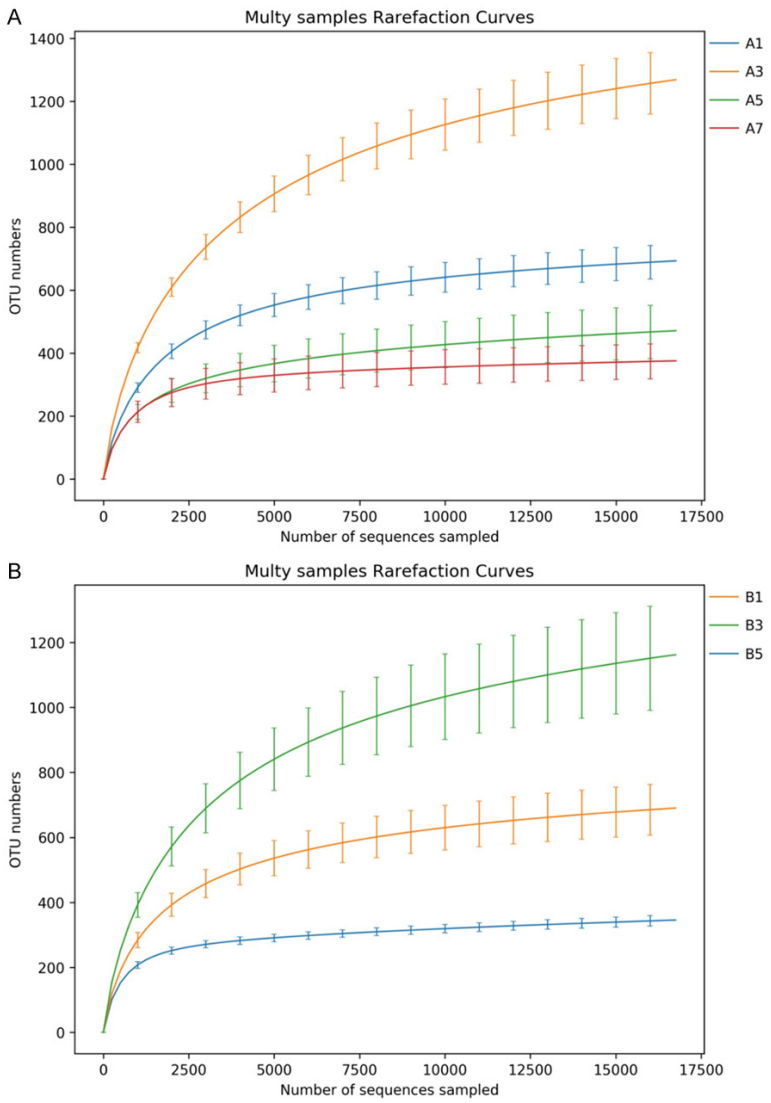


**Figure 1.** Rarefaction curves of each sampling groups. A: Rarefaction curves derived from alive patients; B: Rarefaction curves derived from dead patients.

**Table 2.** Taxonomy distribution of Group A at phylum level

| Phylum | A1 | A3 | A5 | A7 | other |
|---|---|---|---|---|---|
| Proteobacteria | 24.22% | 25.61% | 33.32% | 37.41% | 22.47% |
| Cyanobacteria | 23.87% | 5.24% | 22.26% | 25.35% | 21.18% |
| Bacteroidetes | 14.38% | 30.29% | 15.58% | 14.19% | 19.53% |
| Firmicutes | 19.68% | 23.57% | 14.48% | 13.91% | 18.35% |
| Actinobacteria | 7.63% | 4.28% | 5.13% | 4.97% | 5.30% |
| Acidobacteria | 3.16% | 3.23% | 1.80% | 1.33% | 5.42% |
| Chloroflexi | 1.73% | 1.69% | 1.83% | 0.52% | 2.00% |
| Gemmatimonadetes | 1.09% | 1.09% | 0.65% | 0.32% | 0.87% |
| Verrucomicrobia | 0.54% | 0.83% | 0.41% | 0.36% | 0.69% |
| Deinococcus-Thermus | 0.14% | 0.07% | 1.61% | 0.07% | 0.26% |
| Others | 3.51% | 4.09% | 2.88% | 1.56% | 3.90% |
| Unknown | 0.05% | 0.02% | 0.05% | 0.01% | 0.03% |

On the other hand, when looking into the taxonomic changes chronologically in Group A, noticeable alterations were discovered in the time sequence, especially for Day 3. The proportions of the Cyanobacteria phylum descended distinctly from 24% to 5%, while an arresting 14% to 30% upsurge was observed in the frequency of the Bacteroidetes phylum from Day 1 to Day 3 (**Table 3**). Noticeably, the frequencies of both mentioned phyla regained their previous states in the following days. The ratio of the most frequent Proteobacteria phylum, which was 24% among Group A1, increased steadily to 37% during the 7-day disease course. In term of Group B, similar tendencies were found except for the remarkable changes in Bacteroidetes, which fluctuated around 20% on Day 1 and Day 3 then decreased to 11% thereafter (**Table 4**).

To get further insight about the microbiologic alteration trend during the septic disease course, Beta Diversity Analysis, including Analysis of Similarities (Anosim), Principal Coordinates Analysis (PCoA) and Heatmap, were utilized to explore the taxonomic and abundant variations between samples in different groups. It was revealed from the Anosim analysis that statistical significance was detected between different days among Group A (R=0.149, *p* value =0.001), while the distinction within Group B (R=0.122, *p* value =0.054) was relatively more indeterminate (**Figure 3**). The subsequent PCoA and Heatmap analysis showed that the A3 group tended to be sepa-

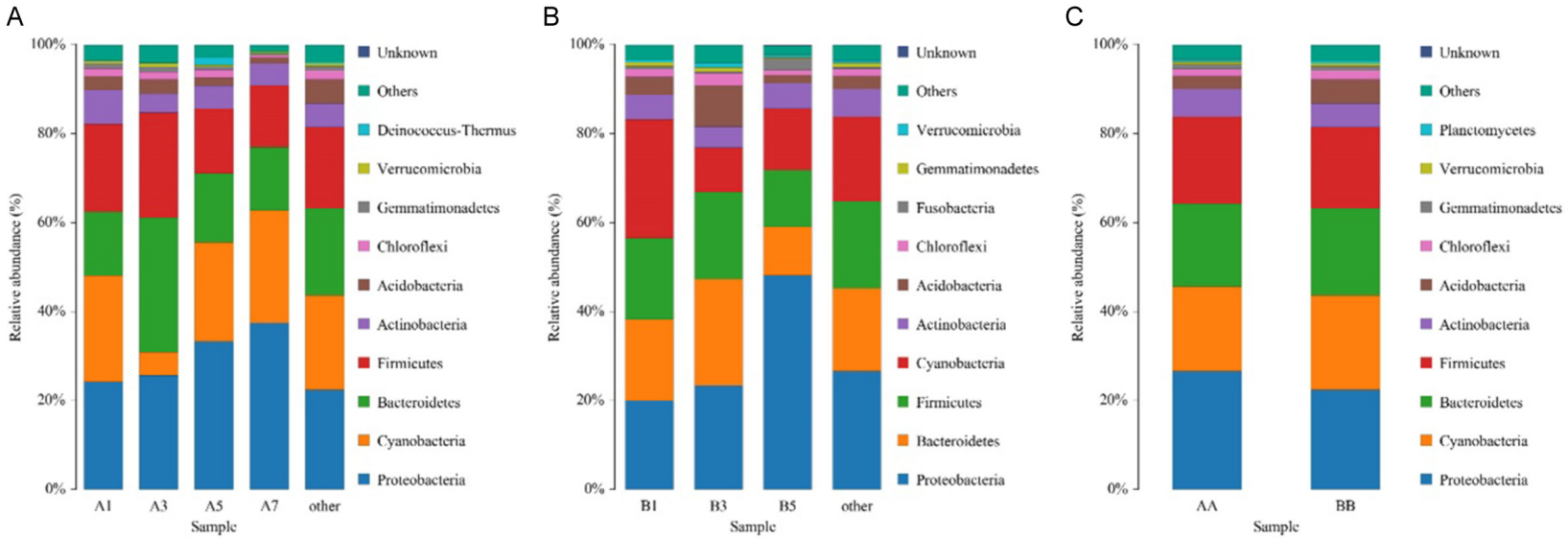


**Figure 2.** Comparisons of phyla abundances in the studied groups. A: Phyla abundances derived from alive patients; B: Phyla abundances derived from dead patients; C: Comparisons of phyla abundances between different groups.

**Table 3.** Taxonomy distribution of Group B at phylum level

| Phylum | B1 | B3 | B5 | other |
|---|---|---|---|---|
| Proteobacteria | 20.02% | 23.36% | 48.23% | 26.64% |
| Bacteroidetes | 18.27% | 23.97% | 10.90% | 18.57% |
| Firmicutes | 18.26% | 19.57% | 12.69% | 19.59% |
| Cyanobacteria | 26.68% | 9.98% | 13.90% | 19.01% |
| Actinobacteria | 5.51% | 4.74% | 5.69% | 6.27% |
| Acidobacteria | 4.02% | 9.24% | 1.80% | 2.88% |
| Chloroflexi | 1.75% | 2.74% | 1.13% | 1.66% |
| Fusobacteria | 0.66% | 0.29% | 2.64% | 0.30% |
| Gemmatimonadetes | 0.87% | 1.00% | 0.23% | 0.98% |
| Verrucomicrobia | 0.56% | 1.01% | 0.53% | 0.58% |
| Others | 3.36% | 4.06% | 2.08% | 3.47% |
| Unknown | 0.02% | 0.03% | 0.18% | 0.04% |

**Table 4.** Comparison of total taxonomy distribution between Group A and Group B at phylum level

| Phylum | AA | BB |
|---|---|---|
| Proteobacteria | 26.64% | 22.47% |
| Cyanobacteria | 19.01% | 21.18% |
| Bacteroidetes | 18.57% | 19.53% |
| Firmicutes | 19.59% | 18.35% |
| Actinobacteria | 6.27% | 5.30% |
| Acidobacteria | 2.88% | 5.42% |
| Chloroflexi | 1.66% | 2.00% |
| Gemmatimonadetes | 0.98% | 0.87% |
| Verrucomicrobia | 0.58% | 0.69% |
| Planctomycetes | 0.49% | 0.53% |
| Others | 3.27% | 3.62% |
| Unknown | 0.04% | 0.03% |

The most frequent 10 phyla are listed.

rated from other samples, and its discreteness also appeared to be lower than any other groups in the PCoA plot (**Figures 4**, **5**).

Line Discriminant Analysis Effect Size (LEfSe) analysis was used to find the putative bacterial biomarkers that may discriminate the characteristics of the sampled groups. The result of Group A was further narrowed by retaining the data derived from the same patients, who were successively sampled at every time point, and eliminating the intermittent samples. Since there were only 2 samples available within B5 group, the filter was not implemented for Group B in case the analyzing volume became too limited. As shown in **Figure 6**, the proposed biomarkers for A1, B1 and A5, and B5 groups had some overlap (order to species of Bromus Tectorum for Day 1, family of Burkholderiaceae for Day 5), while groups of Day 1 showed more similarities to each other (**Figure 7**). On the contrary, the results of A3 and B3 were entirely different with no shared biomarkers. The following comparison between the two groups further confirmed the differences, with Bacteroidetes phylum to Bacteroidales order specific to A3 and Acidobacteria phylum and Rhodospirillales order (belongs to Proteobacteria phylum) labelling B3 (**Figure 7**).

## Discussion

Among patients suffering from severe sepsis or septic shock, positive blood cultures can be obtained in only limited cases despite a proven underlying rate of bacterial infection of 33% [30-32]. This is partly due to technical deficiencies in blood culture acquisition, while it also results from uncultivable microorganisms or very low rates of viable bacteria in the circulating bloodstream [7]. Therefore, it has been speculated that a molecular approach with higher sensitivity for sepsis might overcome the mentioned restrictions of classic microbiologic diagnostics. NGS-based diagnostic testing might offer several remarkable advantages. Along with the increasing importance of NGS in clinical microbiological fields like strain typing, some reports of NGS-based analysis of clinical specimens have been published [33-37]. Currently, there are several published studies focusing on the diagnostic value of NGS technique among clinical sepsis cases [15, 16, 38-41]. Thereinto, Gosiewski et al. used NGS for the analysis of blood samples from 23 healthy volunteers and 62 septic patients to compare the bacterial taxonomic profile [16]. Grumaz et al. aimed at the establishment of a diagnostic workflow for the identification of infectious pathogens from septic patients based on a single-center unbiased sequence analyses of free circulating DNA from plasma by NGS [15]. In another study, Long et al. tested 78 plasma samples from ICU patients using both NGS and BC methods to compare the accuracy and efficiency of the two methodologies, and define the diagnostic threshold of the NGS method [14]. Additionally, other published studies have also demonstrated the value of 16S-amplicon-based or metagenomic

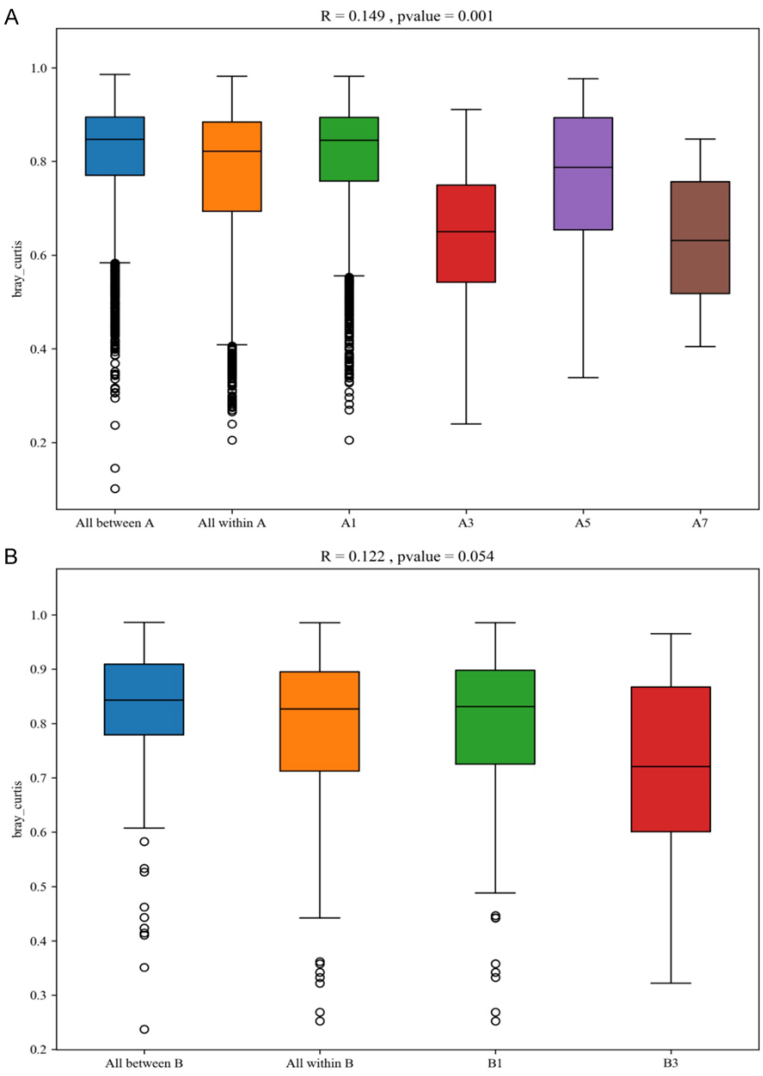


**Figure 3.** Significance test of difference was analyzed using Analysis of Similarities (Anosim). A: Significance test of difference between and within Group A (R=0.145, *p* value =0.001) was analyzed; B: Significance test of difference between and within Group B (R=0.122, *p* value =0.054) was analyzed.

sequencing for diagnosing sepsis and identifying pathogens in the initial stages [13, 17, 38, 39, 42].

On the other hand, instead of further optimizing the analytical process for applying NGS as a microbiologic diagnostic implement, our research was targeted at the supervisory as well as the prognostic potential of the technology among patients with sepsis. In this study, 168 blood samples from 72 septic patients with different survival outcomes were analyzed through NGS at 4 different time points. The chronological monitoring of the patient group enabled more insight into the microbiologic and etiological alterations during the disease process.

As demonstrated above, neglecting the different sampling times, the overall microbial structure of the survival and non-survival groups were not significantly distinct. Nevertheless, it is worth noting that the Actinobacteria phylum, which ranked at fourth with a rate of about 5%, was supposed to be the most frequent phylum among healthy people [16]. According to prior research, the frequency of Actinobacteria significantly dropped from 76.3% in healthy volunteers to 31% in septic patients, while that of Proteobacteria surged from 16.4% to 60.1% [16]. Our observation, was not as dramatic (6% and 5% for Actinobacteria, 26% and 22% for Proteobacteria, respectively), but showed a similar trend in accordance with the published study.

It can be summarized from the successive analysis that the results of Day 3 (including A3 and B3 groups) displayed remarkably distinctive character in contrast to all the other groups. The alterations could be observed from both survival-based groups in Day 3, with the differences demonstrated by all the taxonomic and Alpha-diversity methods. Furthermore, as for the variation from Day 1 to Day 3, the varying degree of Group A (between A1 and A3) appeared to be more obvious than that of Group B (between B1 and B3), which could be revealed from the results of the three types of Beta Diversity analysis. The further LEfSe analysis expressed the same trend as well, while it also manifested a distinction between A3 and B3 through presenting their diverse discriminative biomarkers.

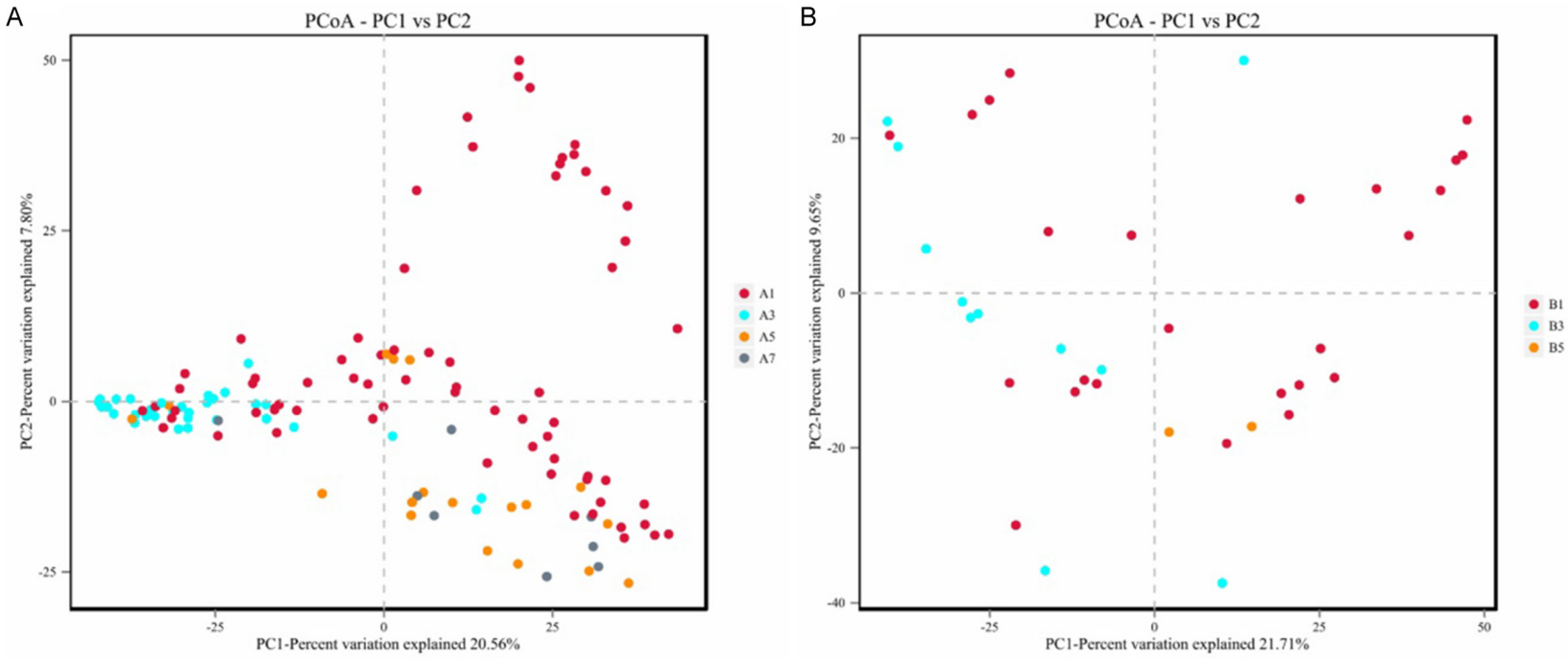


**Figure 4.** Principal Coordinates Analysis (PCoA) plot based on Bray-Curtis derived from NGS sequencing. A: Principal Coordinates Analysis (PCoA) plot based on Bray-Curtis derived from NGS sequencing from samples of group A (A1, n=72; A3, n=33; A5, n=18; A7, n=8); B: PCoA plot based on Bray-Curtis derived from NGS sequencing from samples of group B (B1, n=24; B3, n=11; B5, n=2).

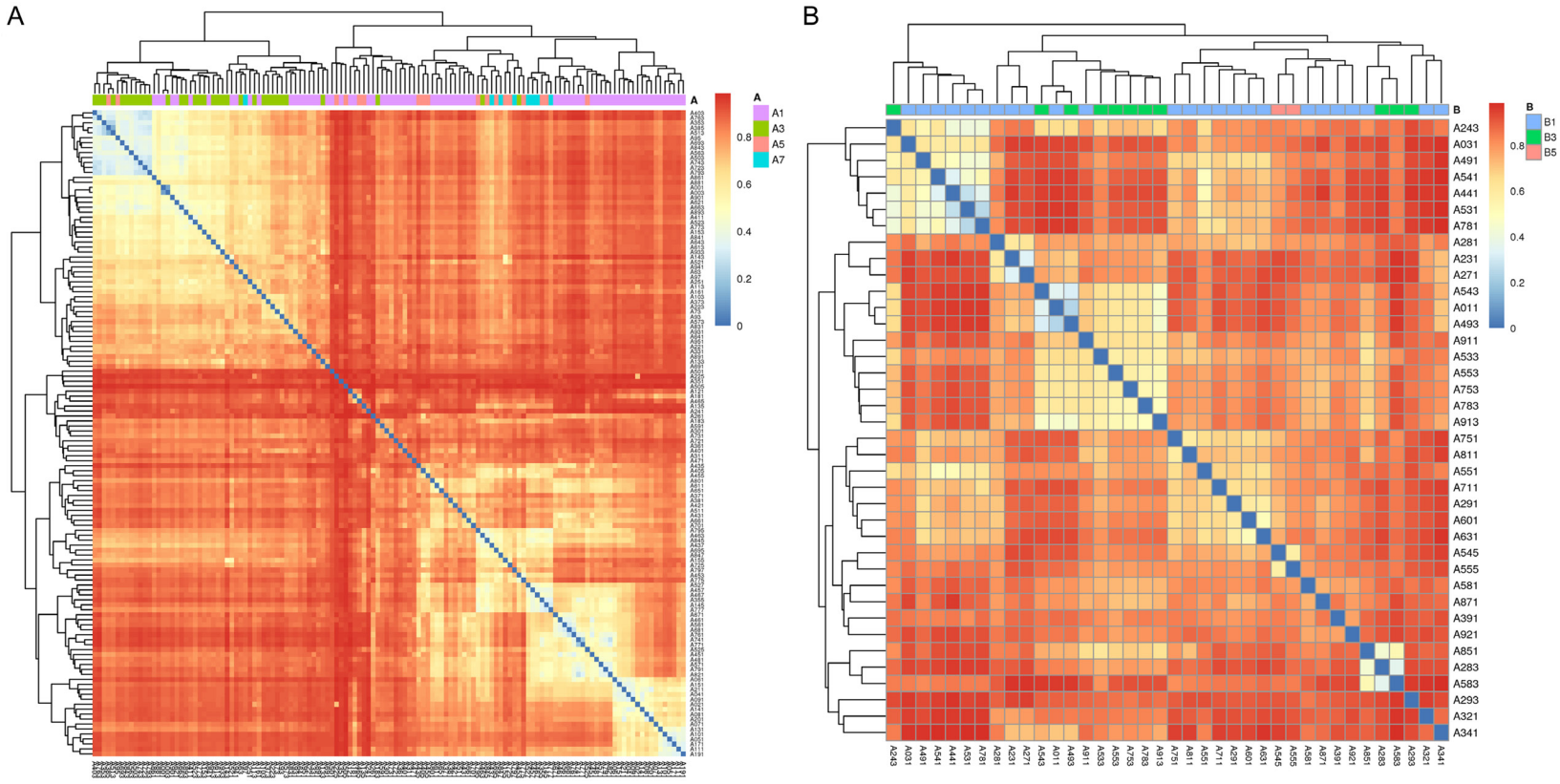


**Figure 5.** Heatmaps based on Bray-Curtis showing the similarity and clustering characteristics. A: Heatmaps based on Bray-Curtis showing the similarity and clustering characteristics of Group A; B: Heatmaps based on Bray-Curtis showing the similarity and clustering characteristics of Group B.

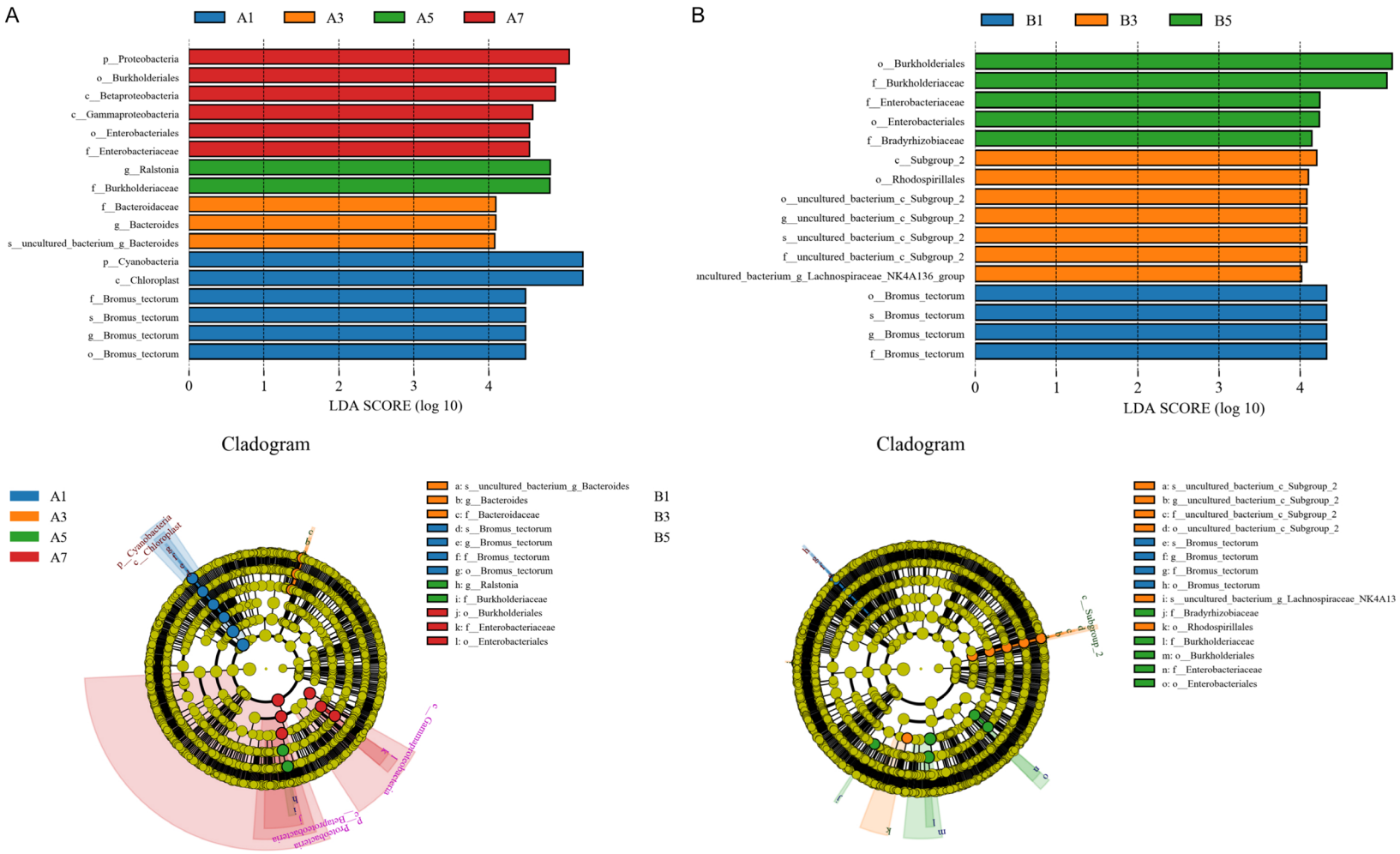


**Figure 6.** Line Discriminant Analysis Effect Size (LEfSe) analysis demonstrating the putative microbiological biomarkers (LDA Score >4). A: LEfSe analysis demonstrating the putative microbiological biomarkers of Group A (6 for A1, 3 for A3, 2 for A5, 6 for A7, respectively); B: LEfSe analysis demonstrating the putative microbiological biomarkers of Group B (4 for B1, 7 for B3, 5 for B5, respectively).

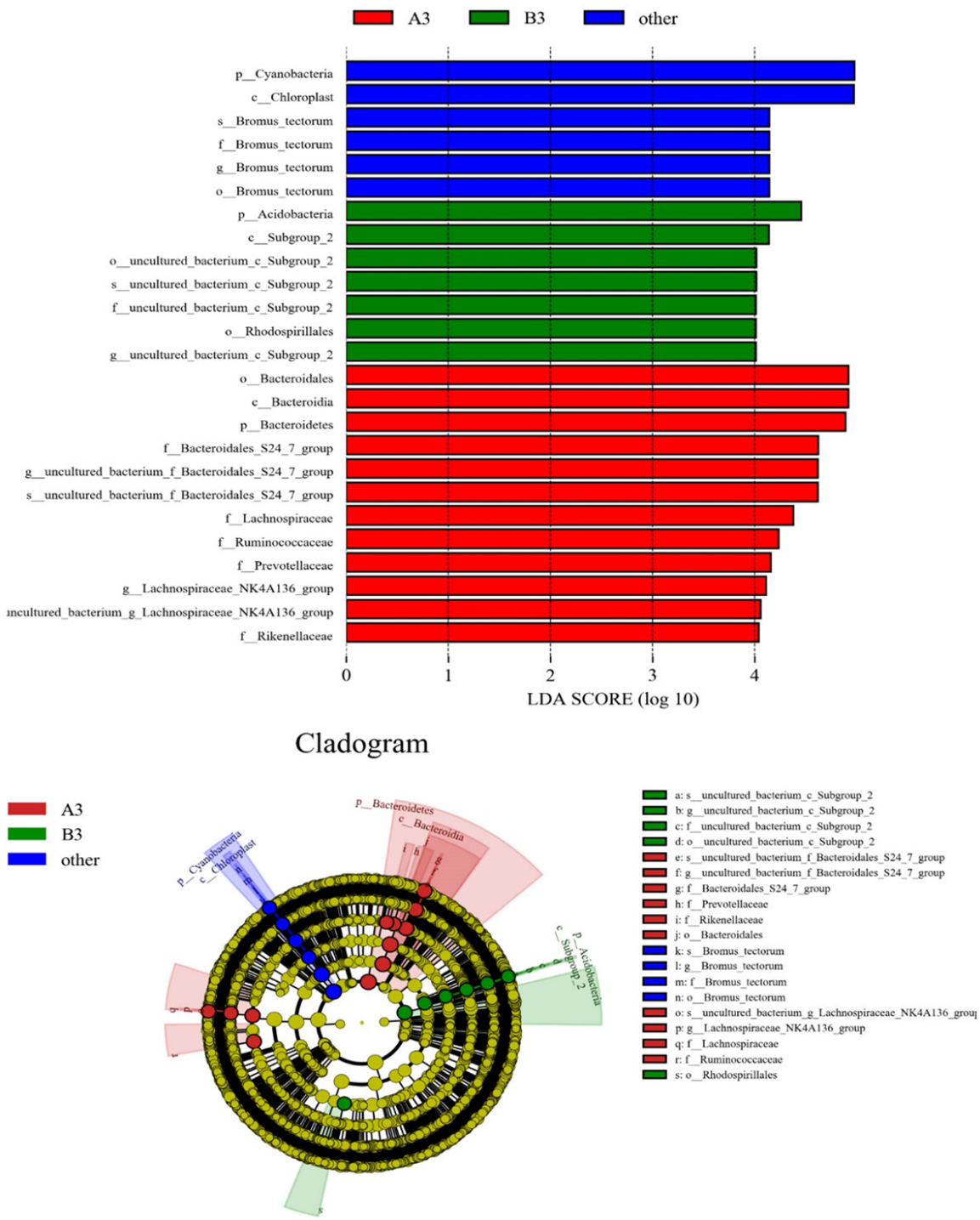


**Figure 7.** Line Discriminant Analysis Effect Size (LEfSe) analysis between A3 and B3 showing the specific proposed biomarkers (LDA Score >4) for each group.

In order to reasonably interpret the phenomenon, interferences from personal and operational error should be excluded in priority. For the sampling and sequencing procedures, the collections of the samples happened at the same site on sporadic dates during a 6-month period, which minimized the influence from single-shot observation. DNA extraction and sequencing of all the samples were carried out at once, in case the divergence between groups could be generated from operative miss or contamination. In this regard, it is worth mention-

ing the presence of several organisms that were commonly deemed as environmental microbes, such as the genera of Bromus tectorum and Ralstonia [43-45], especially when they were detected in abundance among tested samples. Nonetheless, the loads of these microorganisms were so markedly distinct in different sampling stages that they were even respectively suggested as specific biomarkers for Day 1 and Day 5 (**Figure 6**). Considering the operational consistency as mentioned above, the differences between date groups cannot be simply explained by contamination through the process.

Given the premise that the samples within Day 1 showed no significant discrepancy between survival and non-survival groups, the particular microbial changes through the course of the disease were therefore noteworthy.

In terms of predicting the changes and outcome of sepsis, the NGS approach can be regarded as a tool to monitor the alterations from a microbiological perspective. For instance, if a baseline of a certain patient could be established based on genomic result of prior-treatment status, it would be possible to estimate the clinical state through the specific changes during antibiotic therapy [15, 16, 46]. Furthermore, because of the bifurcation between different survival groups since Day 3, a molecular analysis may supplement prognosis, aside from clinical symptoms and examinations.

Nevertheless, it could be revealed from the taxonomic fluctuations that the alterative tendency of microbial constructs within human blood were not necessarily related to their prognostic direction. To be specific, although oriented to diverse survival results, the proportion of Proteobacteria phylum, which is putatively recognized as one of the etiological resources, kept increasing gradually during the course in both Group A and Group B. The nonconformity between bioinformatic and clinical findings could partly due to the existence of cell-free DNA from dead organisms [13]. The capability of detecting inactivated bacteria is acknowledged as a main diagnostic advantage for a genomic method compared to traditional blood culture [13-15, 42], but it may become a pitfall for its prognostic application. In this case, methodological improvements, such as reverse transcription polymerase chain reaction (RT-PCR) technology, should be tried to attempt to eliminate the distraction from devitalized pathogens.

In addition, there are various autologous elements, like immune or metabolic factors, that might affect the microbiological genomic result [47-51]. Hence, the conspicuous alteration of bacterial taxonomic composition on Day 3 may hint at the significance of the time point in the whole progress of the disease, which deserves further investigation from pathophysiological and immunological perspectives. Also, the hypothetical prognosis-conclusive role of the early antimicrobial-treatment stage suggests to clinicians about the importance of close and continuous monitoring during this period [52, 53].

Development of NGS technology enables a thorough microbial investigation for blood-stream infections like sepsis. In this study, the bacterial DNA profiling of blood samples provided several novel findings. The molecular profiling data revealed significant variations between different survival groups in the pathogenesis of sepsis. Our study brings about the probable decisive prognostic value of microbiological monitoring during the early stage of blood-stream infections, which could serve as a practical complement for evaluating the effectiveness of therapeutic regimens in clinical settings. Moreover, there was a distinct presentation of bacterial community in septic blood samples at different disease-progression time points.

## Disclosure of conflict of interest

None.

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