Characteristics of Gut Microbiome and Prediction of Infection in Neutropenic Children with Acute Leukemia

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Abstract

**Background:** Neutropenia in children with acute leukemia have a high incidence of infection and mortality. To identify and classify the potentially infected pathogens, this study compared the structural characteristics of gut microbiome in neutropenic and non-neutropenic children with acute leukemia.

**Results:** The results showed that 6033 OUTs were observed in total, and the sequence coverage index was more than 0.97. In the analysis of alpha diversity, the colony richness index (Chao1 index) of Group A1 was significantly lower than that of Group A0 (P = 0.035). The fecal bacterial communities were dominated by the phylum Firmicutes, Proteobacteria, and Bacteroidetes in both groups, with no significant difference. Higher relative abundance of genera Enterococcus (P = 0.0076), Streptococcus (P = 0.014) and species Bacteroides fragilis (P = 0.034) were observed in Group A1, but class Clostridia (P = 0.038), genera Blautia (P = 0.021) and Roseburia (P = 0.011) were more prevalent in Group A0. The relatively high abundance of Bacteroides fragilis in neutropenia with childhood acute leukemia was an independent risk factor for infection (P=0.028, 95% CI 1.024-1.241).

**Conclusions:** The increase of Enterococcus, Streptococcus and Bacteroides fragilis, and the decrease of Clostridium, Blautia, and Roseburia may be the characteristics of intestinal flora in patients with acute leukemia. The relatively high abundance of Bacteroides fragilis in neutropenia with childhood acute leukemia may predict the occurrence of infection.

**Background**

Leukemia is the most common malignant tumor in childhood and can heal by chemotherapy. In recent years, with the gradual improvement of chemotherapy regimens and the progress of individualized treatment, the complete remission rate and long-term disease-free survival rate of childhood acute leukemia has been significantly improved(1). Infection is part of the most important factors causing the death and treatment failure of childhood leukemia therapy(2-4). Studies have shown that neutrophil deficiency after intensive chemotherapy in leukemia is closely linked to infection-related complications(5). A study by Bent-Are Hansen et al found that patients receiving chemotherapy had higher early mortality in neutropenia with fever(6). Therefore, minimizing the occurrence of infection, identifying the pathogen as soon as possible after the occurrence of infection, and achieving precise treatment is an urgent problem that needs to be solved clinically. Bloodstream infection is a major cause of treatment-related death in childhood leukemia and neutropenia(6). Clinicians often diagnose and treat bloodstream infections according to the symptoms and signs of patients. However, due to the similar clinical manifestations of different pathogens, it is difficult to determine the pathogen of infection clinically, which leads to the delay in diagnosis and treatment. The gold standard for the diagnosis of bloodstream infections is to obtain positive pathogens from blood culture. But this method is slow in detection speed, unable to quantify, and has a high false-negative rate. In current treatment, when neutropenia occurs in childhood leukemia after chemotherapy and there is no sign of infection, antibiotics are often used prophylactically. As we all know, conventional preventive use of antibiotics is
prone to produce resistant strains and even double infections which ia possibly failed to cover pathogenic bacteria and delayed treatment(7). Therefore, for achieving precise treatment, it is urgent to solve the clinical problem that how to minimize the occurrence of infection and identify the pathogen after infection occurred as soon as possible.

Gut microbiome is a complex and dynamic microbial community, which exerts an extremely important influence on the host to maintain body homeostasis and promote disease processes. There are hundreds of billions strains in the human gastrointestinal tract, with more than 1,000 species(8). Among them, the number of intestinal bacteria is 10 times the number of human cells, and the genome content is 100 times that of the human genome (9). Intestinal epithelial cell barrier is a natural barrier to maintain the symbiosis of gut microbiome and host immune cells (10). When the gut microbiome is disordered, the mucosal barrier function decreases, with the intestinal permeability increasing, which can lead to the invasion of a large number of pathogenic microorganisms. Leukemic patients with intestinal mucus layer thinning or even disappearing, as well as intestinal flora disorder, will lead to weak intestinal epithelial mucosal barrier and low systemic resistance, resulting in infection finally(11). Besides, gut microbiome can promote the secretion of IgA in the intestinal mucosa, which can be combined with viruses and bacteria. Therefore, it has a protective role in anti-infection (12). The colonization and rapidly growth of harmful bacteria and conditional pathogenic bacteria, with the reduction of biological barrier function, will enhance the human invasion of pathogenic bacteria and lead to the occurrence of infection. Studies have revealed that most of the infections in patients with hematological malignancies originate from the intestine. The imbalance of gut microbiome in childhood leukemia is an independent risk factor for infection complications (13). The application of chemotherapy and antibiotics will damage the intestinal mucosal barrier of the children, make the intestinal flora losing the interface of colonization and survival and promote the transfer of the flora from the intestine to the blood (14), which cause gut microbiome imbalance, with the decrease of probiotics and the increase of pathogenic bacteria, leading to aggravation of infection and even death in children undergoing chemotherapy eventually.

The 16SrRNA gene sequence, recognized as a target molecule for bacterial species identification, has been widely used (15). The real-time quantitative 16S rRNA PCR amplification system developed by the Maastricht University Medical Center in the United States, which uses the multi-probe assay with a unified probe plus a species or genus-specific probe, can quickly identify several common bacteria in blood infection, including gram-negative bacteria such as Pseudomonas, Escherichia coli, gram-positive bacteria such as Staphylococcus, Enterococcus, Streptococcus and so on within 2 hours (16).

At present, few studies have researched the characteristics of gut microbiome in childhood acute leukemia when neutrophil deficiency occurs during chemotherapy. Childhood acute leukemia during chemotherapy has a high incidence of infection. Early identification of pathogens and early targeted anti-infection treatment will reduce the fatality rate from infection after chemotherapy. In this study, 16SrRNA gene amplicon sequencing technology was used to explore the characteristics of the gut microbiome in childhood acute leukemia with neutropenia, and to identify and classify potential infectious pathogens,
so as to predict the risk of infection in childhood acute leukemia during chemotherapy and provide evidence for the diagnosis of pathogenic bacteria of infection.

**Results**

**Demographic and clinical characteristics of childhood acute leukemia**

Clinical characteristics, patient classifications, infection outcomes, antimicrobial administration are shown in Table 1. The study subjects included 10 specimens of children with AML and 56 specimens of children with ALL, aged 2-15 years (median age 4.6 years), including 34 boys and 32 girls. The study was divided into neutropenia group (Group A1) and non-neutropenia group (Group A0). Antibiotic exposure was identified as patients taking antibiotics orally or intravenously within 2 weeks before specimen collection. Antibacterial drugs were further divided into carbapenems, vancomycin, cephalosporins, penicillins, macrolides, sulfamethoxazole, and itraconazole.

**Sequence analysis of fecal bacteria of children with acute leukemia**

In this experiment, a total of 66 stool samples of children were collected to high-throughput sequencing, and a total of 2,716,105 sequences were obtained by sequencing. The rarefaction curves of all samples (Figure 1A) showed a gradual and flat trend, indicating that the number of sequencing was enough. The Goods coverage index of all samples was higher than 99.7 percent, indicating that the depth of sequencing was sufficient. A total of 6033 operational taxonomic units (OTUs) were calculated by clustering 97% similarity sequences, and then species annotation was carried out based on taxon. The rank-abundance curve (Figure 1B) is mainly used to show the diversity of samples. In the horizontal direction, the width of the curve reflects the abundance of species. The larger the range the curve crosses on the horizontal axis, the higher the species richness is. The smoothness of the curve reflects the average of the species in the sample. The smoother the curve, the more uniform the species distribution.

**Analysis of differences in intestinal microecological diversity between two groups**

**Alpha diversity between two groups**

Alpha diversity, a comprehensive indicator reflecting the abundance and uniformity, refers to the diversity within a specific area or ecosystem. Alpha diversity is mainly related to two factors: one is the number of species, that is, richness, including Chao index and Ace index; the other is the uniformity of individual distribution in the community, that is, diversity, including Shannon index and Simpson index. As shown in Figure 2, the average values of Chao1 index ($P = 0.035$), ACE index ($P = 0.031$), observed OTUs ($P = 0.03$) in Group A1 were significantly lower than those in Group A0. In terms of diversity, the Shannon index of Group A1 was lower than that of Group A0, but it was not statistically significant ($P = 0.15$). This shows that the gut microbiome of childhood acute leukemia with neutropenia is lower than that of non-neutropenic leukemia, which gives an explanation for the high infection rate of the former after chemotherapy.
Beta diversity between two groups

Beta diversity is based on the evolutionary relationship and abundance of information between different sample sequences to calculate the distance between samples. It also describes the similarity and differences between different samples.

Combined with the results of NMDS analysis (Figure 3), it can be seen that the samples of Group A0 and A1 are distributed in two relatively independent regions. Remove the three distant points in Group A1, the remaining samples are in the blue circle, and the samples in Group A0 are in the red circle. After this treatment, the distribution of individuals in Group A1 was more concentrated than that in Group A0, indicating that the gut microbiome between the samples in Group A1 was more similar and the composition of the flora was more monotonous. What's more, it suggests that its resistance to pathogenic bacteria is weaker than Group A0.

Distribution characteristics of gut microbiome in childhood acute leukemia in two groups

In the gut microbiome of all samples, a total of 18 phyla, 35 classes, 58 orders, 103 families, 162 genera, and 80 species were identified. The Rank-Abundance curve shows a gradually flat trend as the sequencing depth increases (Figure 1B). At the phylum classification level, Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are the most important flora (Figure 4A). At the genus level, Group A1 has 11 higher-abundant bacteria while Group A0 has 14 (the average sequencing volume accounts for more than 1% of the total DNA sequencing) (Figure 4B).

Analysis of differences in intestinal microbes between two groups

To explore the characteristics of gut microbiome in childhood acute leukemia with neutropenia, we compared the relative abundance of a certain species between the two groups, and obtained the species causing the difference in the composition and structure of flora between group.

At the phylum level, the content of Firmicum in Group A1 is lower than that in Group A0, while the abundance of Bacteroides is higher. Therefore, the ratio of Firmicum / Bacteroides in the former group is lower than that in the latter group. Although there is no statistically significant difference, there is a tendency to expand the sample size to reach it. As shown in Table 2 and Figure 5, higher relative abundance of genera Enterococcus (p=0.0076), Streptococcus (p=0.014) and species Bacteroides fragilis (p=0.034) were observed in Group A1, while class Clostridia (p=0.038), genera Blautia (p=0.021) and Roseburia (p=0.011) were more prevalent in Group A0.

Gut microbiome predicted of infection in neutropenia with childhood acute leukemia

Table 3 shows the results of binary logistic regression analysis. Dependent variable: whether an infection event occurred; independent variables: Clostridia, Blautia, Faecalibacterium, Ruminococcus, Enterococcus, Streptococcus, Bacteroides fragilis. The P-value of Bacteroides fragilis is 0.028 (95% CI 1.024-1.241). The results show that the relatively high abundance of Bacteroides fragilis in childhood
acute leukemia and neutropenia is an independent risk factor for infection. EXP(B) greater than 1 is a risk factor, and less than 1 is a protective factor. The EXP(B) of Bacteroides fragilis is 1.241, indicating that the risk factor is 1.241 time. That is, the relatively high abundance of Bacteroides fragilis in neutropenic childhood acute leukemia can predict the occurrence of infection.

**Discussion**

Childhood acute leukemia has become the second mortality disease in China. Currently, chemotherapy is the main treatment, and the long-term survival rate of this disease after chemotherapy can amount to 85%(17, 18). However, children with leukemia after chemotherapy inevitably suffer from bone marrow suppression and neutropenia. At the same time, because of the abnormal white blood cell function and followed with lose of normal human resistance, thus increases the chance of infection. Intestinal mucosal barrier mainly includes mechanical barriers, chemical barriers, immune barriers, and biological barriers. The principal function is to prevent bacteria translocation and endotoxin absorption(19). Stringer, A. Met al found that chemotherapy can induce the synthesis of reactive oxygen species (ROS), free radicals and ceramides, which can lead to cellular DNA damage, intestinal mucositis, and activate nuclear transcription factor (NFκB). It can also produce many pro-inflammatory factors to increase intestinal inflammation, break the intestinal microecological balance, damage the intestinal mucosal barrier function, which leads to mass propagation and translocation of opportunistic pathogens, harmful bacteria invading, and eventually causing infection(20).

Most studies have shown that most of the infections in patients with hematological malignancies originate from intestinal flora. However, few studies have been conducted on the composition and structure of intestinal flora in childhood acute leukemia and the relationship between infection and infection outcomes. In this study, a total of 66 fecal specimens of childhood acute leukemia were collected, of which 16 were neutropenia and 50 were non-neutropenia. Aarne Kolonen's study found that bloodstream infection affected the prognosis of childhood acute leukemia receiving intensive treatment. The results showed that there were 977 courses of blood culture data, of which 503 cases of bloodstream infection (51%), and 20 cases died of infection (5.6%) ), of which 16 were bloodstream infection-related deaths (80%) (21). A total of 29 infection events occurred in our study, the infection rate was 43.9%, and no death occurred, which was lower than the infection rate and mortality rate of Aarne Kolonen's study. Previous studies have found that neutropenia in childhood acute leukemia has a higher incidence of infection(5). In this study, the infection rate of childhood acute leukemia with neutropenia was 87.5% (14/16), while the infection rate of that without neutropenia was only 30% (15/50). By comparing the structural characteristics and differences of the intestinal flora of the two groups, this study is expected to find potential infectious pathogens and use targeted antibiotics early, thereby shortening the length of hospital stay, reducing the progression of severe infections to systemic inflammatory response syndrome, and even death happened. Our results showed that Alpha diversity index (Chao1 index P = 0.03, Shannon index P = 0.15) in neutropenic with childhood acute leukemia was significantly lower than that in non-neutrophilic with childhood acute leukemia children. Previous studies have also found that the microbial diversity of intestinal flora in children with ALL, AML or non-Hodgkin's lymphoma (NHL) decreased during
chemotherapy, and gradually recovered with the extension of chemotherapy (13, 22, 23). Early studies found that the diversity of intestinal flora in childhood acute lymphoblastic leukemia was significantly lower than that in healthy controls at baseline (24). There were also significant differences in intestinal flora diversity among childhood acute lymphoblastic leukemia who had recently used antibiotics or not (25). We can expand the sample size, track the changes in the diversity of intestinal flora during the chemotherapy-induced remission, consolidation, and maintenance phases of children, and predict the likelihood of infection based on their baseline, which is our next goal.

Previous studies have shown that Firmicutes, Bacteroidetes, Actinomycetes, and Proteobacteria constitute the vast majority of intestinal flora, of which the former two account for more than 90% (26). In addition to the Firmicutes and Bacteroidetes, the proportion of Proteobacteria is also large, in our research, and the combined proportion of the three is greater than 95%. Simultaneously, our study observed that the abundance of Proteobacteria in the intestinal flora of children with neutropenia is higher than that of children with non-neutropenia, which is consistent with the study by Hana Hakim (13), suggesting that the relatively high abundance of phylum Proteobacteria may predict febrile neutropenia. Research by Taur, Ying (27) found that during allogeneic hematopoietic stem cell transplantation, Firmicutes accounts for more than 30%, which can increase the risk of Gram-negative bacilli bacteremia by 5 times. In terms of ecological imbalances, the relative abundance ratio (F/B) of Firmicutes and Bacteroides is a commonly used evaluation index and represents the composition structure of intestinal flora. The Phylum Firmicutes helps the body absorb and store energy from food, while the Phylum Bacteroidetes helps the body consume energy. In some hematological malignancies, such as multiple myeloma (28, 29), Hodgkin's disease (30), the proportion of Bacteroides in the intestine tracts of patients was significantly increased compared with the healthy control group, suggesting that the increase of phylum Bacteroides is related to the high energy consumption state of immune disorder. Most Bacteroidetes are Gram-negative bacteria with an outer membrane containing endotoxin. Related studies have shown that intestinal permeability of patients with multiple myeloma with high-abundant Bacteroides increased (30), which is beneficial to the intestinal flora and toxin product translocation. In this study, the abundance of Firmicium decreased and Bacteroides increased in neutropenic acute leukemia children, and the F/B ratio was lower than that in non-neutrophilic acute leukemia children. It is suggested that neutrophil deficient with childhood acute leukemia have reduced energy intake and storage, and maybe in a state of high energy consumption due to immune disorder, resulting in poor body resistance during bacterial invasion and more prone to infection.

Blautia is a Gram-positive, non motile, spherical, or oval anaerobic bacteria. It produces acetic acid, lactic acid, succinic acid, ethanol, and hydrogen as the main metabolites of glucose metabolism. These metabolic end products, especially the fermentation product butyrate, can enhance the immune function of intestinal mucosa (31, 32). In a cohort study, increased bacterial abundance in Blautia was closely associated with decreased lethal acute graft-versus-host disease (GVHD) and improved overall survival, suggesting that Blautia may result in suppressing immune rejection (33). Blautia is part of the most important markers to improve the prognosis of various clinical diseases such as visceral fat accumulation (34) and Crohn's disease (35). Roseburia bacteria are also a part of symbiotic bacteria that
produce short-chain fatty acids (especially butyric acid), which maintain immunity and have anti-inflammatory properties. Flagellin of R intestinalis may up-regulate the expression of tight junction protein through toll like receptor 5 (TLR5), thus restoring the integrity of intestinal barrier, and helping to restore intestinal flora by increasing the expression of IL-22 and REG3γ(36). Our research found that the abundance of Blautia and Roseburia bacteria in acute leukemia children with neutropenia was significantly reduced, which was negatively correlated with the incidence of infection. The loss of Clostridia members (including Roseburia, E. faecalis, Rumenococcus, and Blautia) is associated with poor prognosis in a variety of diseases. In addition to the aforementioned bacteria of genera Roseburia and Blautia, the abundance of the genus Faecalibacterium and Rumenococcus of acute leukemia children with neutropenia is less than that of non-neutropenia group. These two genera are associated with anti-inflammatory effects and may be involved in maintaining the integrity of the intestinal epithelium(36). There is no significant differences between the two bacteria in statistics, which may be due to the insufficient sample size, which needs to be verified by expanding the sample size.

Enterococcus is a lactate-producing bacteria. When the content of this flora is abnormally increased, the accumulation of lactate can increase the permeability of the intestinal mucosa, which can cause endotoxemia, ulcerative colitis, and even lead to bowel resection(37). Studies have shown that enterococci or Streptococcus are predictors of infection after chemotherapy regardless of the stage of chemotherapy(13). A study by the American Society for transplantation and cell therapy found that 89.3% of patients survived two years after allogeneic hematopoietic stem cell transplantation (HSCT) had less than 1% enterococci abundance, suggesting a significant correlation between low abundance enterococci and high survival rate(38). Our study showed that the abundance of Enterococcus and Streptococcus in neutrophilic with childhood acute leukemia was significantly higher than that in patients with non-neutropenia. It is suggested that the intestinal mucosal barrier of children with neutropenia is more permeable, and it is easy for bacterial flora to shift and absorb endotoxin, resulting in infection outcomes. Bacteroides fragilis belongs to the normal intestinal flora, mainly in the colon. Bacteroides fragilis ranks first in clinical anaerobic bacteria isolation, accounts for up to 20%. When the intestinal mucosa of the host is damaged, Bacteroides fragilis heterotopic becomes an opportunistic pathogen, causing purulent infection of various organs of the body, such as intestine, abdominal cavity, liver, lung, and brain, accompanied by abscess and acute and chronic diarrhea(39). Bacteroides fragilis can secrete enterotoxins, make the adhesion and connection between the cells of human colon epithelial cell line HT29 / C1 disappear, round, and swell the cells. At the same time, it can increase the secretion of chloride ions and inhibit the absorption of sodium ions, thereby weakening the intestinal mucosal barrier and resulting in Bacteria translocation(40, 41). In recent years, Bacteroides fragilis has developed resistance to a variety of commonly used antibiotics, especially imipenem and metronidazole(42, 43). Our study has observed that the abundance of Bacteroides fragilis in neutropenic with childhood acute leukemia is significantly higher than that in children with non-neutropenia. This explains why the infection rate of the former is significantly higher than that of the latter.

In conclusion, the infection rate of acute leukemia with agranulocytosis after chemotherapy was significantly greater than that of non-agranulocytosis group. In the agranulocytosis group, Enterococcus,
Streptococcus, and Bacteroides fragilis increased, while Clostridium, Blautia and Roseburia decreased. However, according to the binary regression analysis, only relatively high abundance of Bacteroides fragilis in the agranulocytosis group can predict the occurrence of infection, and the risk factor is not high, which is considered to be caused by too few samples. We need to further expand the sample size and conduct multi center research, to find out the intestinal flora which can accurately predict the infection in the early stage, and early targeted anti-infection treatment, so as to reduce the infection rate and mortality rate after chemotherapy.

Conclusions

The increase of Enterococcus, Streptococcus and Bacteroides fragilis, and the decrease of Clostridium, Blautia, and Roseburia may be the characteristics of intestinal flora in patients with acute leukemia. The relatively high abundance of Bacteroides fragilis in neutropenia with childhood acute leukemia may predict the occurrence of infection.

Methods

Study population

The study included 29 childhood acute leukemia as subjects, hospitalized in The Second Affiliated Hospital of Shantou University (Shantou, China) from January 2018 to December 2018. All participants fulfilled the inclusion selected criteria by a questionnaire survey and laboratory tests. The subjects were divided into experimental group and control group according to whether they had neutropenia. All participants fulfilled the inclusion selected criteria by a questionnaire survey and laboratory tests: (1) according to clinical symptoms, signs and bone marrow cytology, it is consistent with childhood acute leukemia (including acute lymphoblastic leukemia and acute myeloid leukemia)\(^{(44, 45)}\); (2) blood routine test, liver, and kidney function before chemotherapy are normal; (3) no diarrhea within two weeks before sampling; (4) stool routine examination before chemotherapy is normal; (5) children have not used antibiotics and microecological preparations within 2 weeks before collecting specimens.

Specimen collection

The sample collection procedure has been approved by the Ethics Committee of Shantou University Medical College, and the informed consent of the parents or guardians of enrolled children has been obtained. Fecal samples were collected from 29 children who met the above conditions one day before chemotherapy and when neutropenia occurred after chemotherapy. At least 2g fresh stool was collected with a sterile centrifuge tube, and then immediately stored in -80 °C refrigerator for standby. A total of 66 fecal specimens were collected, 16 of which were in the neutropenic acute leukemia group (experimental group), and 50 were in the non-neutrophilic acute leukemia group (control group).

DNA extraction
The fecal specimen was thawed in a 37°C water baths, and the total DNA was detected by stool DNA Extraction kit (D4015-01; Omega). The total DNA extracted from the above was determined by 1% agarose gel electrophoresis.

**PCR amplification and 16SrDNA sequencing**

Total DNA of gut microbiome obtained in the previous step was used as a template, and primers 338F 5’-ACTCCTACGGGAGGCAGCAG-3’ and 806R 5’-GGACTACHVGGGTWTCTAA T-3’ were used to amplify the highly variable V3-V4 region of 16SrRNA. 25uL reaction system: 50ng template DNA, 12.5uL Phusion Hot Start Flex 2X Master Mix (M0536L; NEB), 2.5uL primers, and PCR grade water for volume adjustment. The amplification procedure is as follows: pre-denaturation at 98 °C for 30 seconds, denaturation at 98 °C for 10 seconds, annealing at 54 °C for 30 seconds, extension at 72 °C for 45 seconds, a total of 35 cycles, and final extension at 72 °C for 10 minutes to end. The PCR product was confirmed by 2% agarose gel electrophoresis. PCR products were normalized by AxyPrep Mag PCR Normalizer (Axygen Biosciences, Union City, CA, USA), which allowed for the skipping of the quantification step regardless of the PCR volume submitted for sequencing. Then, based on the Illumina Miseq platform, the amplified products were constructed and sequenced.

**Bioinformatics analysis**

Raw data obtained after sequencing included dirty reads containing adapters or low-quality bases which would affect the following assembly and analysis. Thus, to get high-quality clean reads, raw reads were further filtered by fastp(0.12.4), according to the following rules: (1) Removing reads containing more than 5% of unknown nucleotides (N); (2) Removing reads containing less than 80% of bases with quality (Q-value)>20. Paired end clean reads were assembled as raw tags using QIIME(1.9.1, with a minimum overlap of 10 bp and mismatch error rates of 2%. Tags were screened to remove chimeras using usearch61 in QIIME(1.9.1), which performs both de novo chimera and reference based detection. The software QIIME(1.9.1) was used to cluster tags of more than 97% identity into OTUs with the open reference method, and then the taxonomy of OTUs was assigned by uclust based on Greengenes Database(13.8). The phylogenetic tree was constructed using FastTree. Between groups, Venn analysis was performed in R to identify unique and common OTUs. According to the OTU's species annotation information, count the number of Tags sequences at each classification level (kingdom, phylum, class, order, family, genus, species) for each sample.

**Statistical analysis**

All experimental data are expressed as mean ± standard deviation. The diversity parameters and structural difference analysis of the microbial community are analyzed by non-parametric statistical analysis using T-test test and Wilcoxon rank sum test. When P <0.05, it is considered to be statistically significant. The statistical software used is STATISTICA V12.5 (STAT SOFT, USA).

**Abbreviations**
AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; Group A₁: neutropenia in children with acute leukemia; Group A₀: non-neutropenia in children with acute leukemia.

Declarations

Ethics approval and consent to participate

The study has been approved by the Ethics Committee of Shantou University Medical College, and the informed consent of the parents or legal guardians of the enrolled children has been obtained.

Consent for publication

Not Applicable.

Availability of data and materials

All datasets generated for this study are included in the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

HW WANG, BX LI, and L MA designed the experiments and drafted the manuscript. HW WANG, AJ LI, P NI, and BX LI performed all the experiments. Y ZHONG, LM LIN, HW WANG and BX LI analyzed the data. All authors read and approved the manuscript.

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Tables

Table 1. Patient Characteristics
### Characteristics

| Characteristics                                                                 | 66 subjects (%) |
|-------------------------------------------------------------------------------|-----------------|
| Diagnose, n(%)                                                                | APL 10 (15.2); ALL 56 (84.8) |
| Age, years, Median (Range)                                                     | 4.6 (2-15)      |
| Age, n (%)                                                                    |                 |
| 2-<5 years                                                                    | 35 (53)         |
| 5-<10 years                                                                   | 18 (27.3)       |
| ≥10 years                                                                     | 13 (19.7)       |
| Sex, n (%)                                                                    |                 |
| Male                                                                          | 34 (51.5)       |
| Female                                                                        | 32 (48.5)       |
| Stool samples, n                                                              |                 |
| Neutropenia                                                                   | 16 (24.2)       |
| Non-neutropenia                                                               | 50 (75.8)       |
| Infection                                                                     | 29 (43.9)       |
| Non-infection                                                                 | 37 (56.1)       |
| Infection rate of children with neutropenia and acute leukemia                | 14/16(87.5)     |
| Infection rate of children with non-neutropenia and acute leukemia            | 15/30(30)       |

### Patients with infection outcomes

| Patients with infection outcomes |  |
|---------------------------------|--|
| Bloodstream infections          | 12 (18.2) |
| Febrile neutropenia             | 7 (10.6)   |
| Respiratory illness             | 18 (27.3)  |
| Other infections                | 5 (7.6)    |
| No infection outcome            | 37 (56.1)  |

### Antimicrobial administration

| Antimicrobial administration |  |
|-------------------------------|--|
| Carbapenems                   | 10 (15.2) |
| Vancomycin                    | 4 (6.1)   |
| Cephalosporins                | 22 (33.3) |
| Penicillins                   | 2 (3)     |
These are infection outcomes of child patients. (note that the numbers of individual infection outcomes add up to >100% because some patients received more than 1 of the listed infection outcomes during chemotherapy).

Clinically defined bloodstream infection (9 episodes), Microbiologically defined bloodstream infection included methicillin-resistant staphylococcus aureus (1), methicillin-resistant coagulase negative staphylococcus (1), staphylococcus epidermidis (1).

Etiologies included otitis media (1 episode), allal fissure (2), lower urinary tract infection (1), infection of unknown origin (1).

No fever, bacteremia, febrile neutropenia, or respiratory illness.

Some child patients were used two or more antibiotics. Cephalosporins: 18 cases of ceftriaxone, 2 cases of ceftazidime, 2 cases of cefuroxime; 2 cases of penicillins (1 case of piperacillin/tazobactam, 1 case of amoxicillin); Macrolides: azithromycin 2 cases.

Table 2 Comparison of the abundance of gut microbiome between two groups

| Class                  | Mean(A0-G20) | Mean(A1-G20) | P-value     |
|------------------------|--------------|--------------|-------------|
| Clostridia             | 43.76011     | 29.75363125  | 0.037543242 |
| Blautia                | 4.555958     | 1.10248125   | 0.021207457 |
| Streptococcus          | 1.79164      | 3.26528125   | 0.013555348 |
| Enterococcus           | 2.814598     | 7.95331875   | 0.00763096  |
| Bacteroides fragilis  | 1.61034      | 6.419775     | 0.033683467 |

Higher relative abundance of genera Enterococcus (p=0.0076), Streptococcus (p=0.014) and species Bacteroides fragilis (p=0.034) were observed in Group A₁, while class Clostridia (p=0.038), genera Blautia (p=0.021) and Roseburia (p=0.011) were more prevalent in Group A₀.

Table 3 Variables in the Equation
|            | B     | S.E.  | Wald | df | Sig.  | Exp(B) | 95% C.I. for EXP(B) |
|------------|-------|-------|------|----|-------|--------|---------------------|
|            |       |       |      |    | Lower | Upper  |                     |
| Clostridia | 0.034 | 0.021 | 2.671| 1  | 0.102 | 1.034  | 0.993   1.077       |
| Blautia    | 0.016 | 0.049 | 0.101| 1  | 0.751 | 1.016  | 0.922   1.119       |
| Faecalibacterium | 0.176 | 0.158 | 1.235| 1  | 0.266 | 1.192  | 0.874   1.625       |
| Ruminococcus | -0.027 | 0.026 | 1.079| 1  | 0.299 | 0.974  | 0.926   1.024       |
| Enterococcus | 0.027 | 0.018 | 2.218| 1  | 0.136 | 1.027  | 0.992   1.064       |
| Streptococcus | 0.085 | 0.060 | 2.045| 1  | 0.153 | 1.089  | 0.969   1.225       |
| Bacteroides fragilis | 0.216 | 0.098 | 4.828| 1  | 0.028 | 1.241  | 1.024   1.505       |
| Constant   | -2.264| 0.790 | 8.222| 1  | 0.004 | 0.104  |                     |

It shows the results of binary logistic regression analysis. Dependent variable: whether an infection event occurred; independent variables: Clostridia, Blautia, Faecalibacterium, Ruminococcus, Enterococcus, Streptococcus, Bacteroides fragilis.

**Figures**
Rarefaction curves(A). The rarefaction curves of all samples showed a gradual and flat trend, indicating that the number of sequencing was enough. Rank-Abundance curves(B). In the horizontal direction, the width of the curve reflects the abundance of species.
Figure 2

Differences in alpha diversity between samples from A0 and A1 patients that were sequenced by 16SrRNA gene sequencing. Significance was determined using a T-test at an alpha value of 0.05 (represented by *). Each panel represents a different measure of alpha diversity; ACE index (A), Chao index (B), Observed OUTs (C), and Shannon index (D). A0: non-neutropenic children with acute leukemia. A1: neutropenic children with acute leukemia.
Figure 3

Beta diversity graph of GI microbiota established by NMDS analysis based on unweighted Unifrac distance. Significance was determined using a T-test at an alpha value of 0.05 (represented by *). A0: non-neutropenic children with acute leukemia. A1: neutropenic children with acute leukemia.
Figure 4

A. The abundance of bacteria at the phylum level. A0: non-neutropenic children with acute leukemia. A1: neutropenic children with acute leukemia. B. The abundance of bacteria at the genus level. A0: non-neutropenic children with acute leukemia. A1: neutropenic children with acute leukemia.
Figure 5

Comparison of the abundance of gut microbiome between group A0 and group A1: Clostridia(A), Blautia(B), Streptococcus(C), Enterococcus(D), Bacteroides fragilis(E).

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- **Originaldata.rar**