ORIGINAL ARTICLE

Exploring the Characteristics of Intestinal Microbiota in Hematologic Malignancy Patients via 16s rDNA High-Throughput Sequencing

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SUMMARY

Background: Malignant hematopathy is an important branch of malignant tumors, with high mortality and malignancy. The purpose of this study was to investigate the relationship between intestinal microecology and diseases by observing patients with newly diagnosed hematologic malignancy.
Methods: In this study a total of 23 stool samples were collected and analyzed, 13 of which were stool samples from newly diagnosed patients with malignant blood system diseases, and 10 were healthy individual controls. The characteristics of the intestinal flora were analyzed through 16s rDNA technology in the next-generation sequencing (NGS).
Results: Bacteroidetes and Firmicutes accounted for the major abundance of the intestinal microecology in both patients with hematological malignancies and healthy controls, whereas the abundance of Bacteroidetes in the patient group was lower than that in healthy controls. Linear discriminant analysis (LDA) in LEfSa was used to search for landmark species, suggesting that Erysipelotrichi, Erysipelotrichales, and *Erysipelotrichaceae* could be considered as markers for patients with hematological malignant diseases. *Butyricicoccus pullicaecorum*, *Bacteroides plebeius*, and *Collinsella aerofaciens* also contribute to potential values as markers for intestinal flora in hematological malignant patients.
Conclusions: Patients with hematologic malignancies have altered intestinal flora structure compared with healthy individuals, which can provide new ideas for the treatment of hematologic malignancies.
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KEY WORDS

INTRODUCTION

Next generation sequencing can detect a large number of target genes at one time with high sensitivity and specificity, showing a broad clinical and scientific application prospect in many fields such as pathogenic microorganisms and metagenomics, tumor gene mutation, and genetic diseases [1,2]. Increasingly, with the development of NGS technology, an interest in gut microbes has emerged. Under normal circumstances, the host maintains a dynamic microecological balance with the

normal flora. The obligate anaerobes bind to specific receptors on the surface of intestinal mucosa to form a stable bacterial membrane structure. Subsequently, it forms a biological barrier that can effectively protect the host from the invasion of bacteria and plays an important role in maintaining intestinal structure and function. Previous studies have shown that intestinal flora interacts with distal organs in the intestinal brain axis [3], the intestinal liver axis [4], the intestinal kidney axis [5], and the intestinal lung axis [6]. Intestinal microbiome therapy may be an effective treatment for certain diseases. In addition to probiotics known to improve the prognosis of inflammatory bowel disease (IBD) [7], fecal microbiota transplantation (FMT) can treat persistent clostridium difficile infections [8] and promote the treatment of phenylketonuria and other genetic diseases [9]. In cancer research, Riquelme E et al. have reported significant differences in the composition of intestinal flora from pancreatic cancer patients with short term survival (STS) versus those with long term survival (LTS). LTS can be accurately predicted by using bacteria enriched in LTS patients. Transplanting STS and LTS fecal bacteria into mice can affect tumor growth and immune infiltration [10]. All of these provide a new idea for tumor treatment. Hematologic malignant oncology is one of the most remarkable subjects in medical research in the world. Blood malignant tumors (hematologic malignancy) belongs to a group of malignant clonal diseases of hematopoietic stem cells which possess the abilities of clonal self-renewal, multi-directional differentiation, and maturation. Although there are a few studies on intestinal flora and malignant blood diseases which suggest intestinal flora changes after hematopoietic stem cell transplantation [11,12], the understanding of the relationship between gut microbiota and malignant hematopathy is still limited. Therefore, the present study tried to harness next generation sequencing technology to investigate the changes in the intestinal flora of patients with the malignancies of the blood system in comparison with healthy controls. Our results indicate that the intestinal flora of patients with hematological malignancies is significantly different from that of healthy controls, as well as specific colonies. This provides a potential basis for better diagnosis and treatment of malignant hematologic tumors and better management of the intestinal flora.

MATERIALS AND METHODS

Sample collection
The study was designed to evaluate the gut microbiota composition in patients diagnosed with hematologic malignancy. In total, samples from 23 individuals divided into two groups were collected from the First Affiliated Hospital of Anhui Medical University, China. In detail, 13 samples were from hematologic malignancy patients before any treatment, which were denoted as group A. Ten samples were from healthy individuals, denoted as group B.

The procedure for collecting stool samples was as follows: First, all the subjects needed age-matched heights and body weights. Next, patients with known intestinal microecology-related diseases such as inflammatory bowel disease, diabetes, and dyslipidemia were excluded. Patients with infectious diseases within the previous month were also excluded from treatment with antibiotics, hormones and other conditions. Last but not least, individuals who recently used prebiotics or probiotics were not included in the study.

All stool samples were collected in sterile containers, which were immediately frozen and stored in a -80°C freezer. This study was approved by the ethics committee of the First Affiliated Hospital of Anhui Medical University. All subjects in this study gave informed consent.

DNA extraction and polymerase chain reaction (PCR) amplification
After the stool samples frozen at -80°C were restored to room temperature, the bacterial DNA of the stool samples was extracted by TIANamp DNA Stool DNA Kit according to the manufacturer's protocol.

The PCR amplification of 16s ribosomal DNA gene was performed in GeneAmp 9700 (AppliedBiosystems, Foster City, CA, USA). The total volume of the PCR reaction system was 20 μL, including 10 μL 5 x PrimeSTAR Buffer, 4 μL dNTP Mixture, 0.5 μL PrimeSTAR HS DNA Polymerase, 1 μL forward primer, 1 μL reverse primer, 10 ng Template DNA, and the rest was supplemented with ddH$_2$O. The forward primer sequence: GTGCCAGCMGCCGCGGTAA, and the reverse primer: CCGTCAATTCMTTTRAGTTT, targeted the 16s v4-v5 region of bacteria. The PCR procedure was as follows: pre-denaturation was performed at 94°C for 5 minutes, followed by 27 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, the final extension was operated at 72°C for 5 minutes. The amplified PCR products were sequenced on Illumina Miseq using the next generation of bridged PCR.

Bioinformatics process and statistical analysis of 16s rDNA data
In order to obtain high quality clean reads, a series of bioinformatics processing on the raw data needed to be performed. First, each piece of the sample data was separated from the raw data according to Barcode sequence and PCR amplification primer sequence. Then the Barcode sequence and primer sequence were cleaved, and the reads of sample with double-ends were spliced, subsequently the Raw Tags were obtained. After the Raw Tags were processed accordingly and the low quality, over-lengthened and chimeric Tags were further stripped out, the final Tags were considered as high-quality Clean Tags. The obtained Clean Tags were clustered with Mothur software. The obtained tags with 97% similarity could be classified as Operational Taxonomic Units (OTUs). Due to ribosomal RNA conservation, se-

quences with high similarity to each other should be classified into the same category, namely OTUs. All of the following analyses were based on OTUs. This study classified sequences on the platform of RDP software and used SILVA database to determine the classification information of a sequence from door to species level, in which the corresponding confidence threshold of each classification level was 0.5.

Shannon dilution curve is primarily used to assess whether the sequencing volume is sufficient and indirectly reflects the richness of species in the samples. When the curve flattens or reaches a plateau, it can be considered that the increase in sequencing depth has no impact on species diversity and the sequencing volume tends to be saturated. Two different algorithms were applied in the present study to investigate the evolutionary relationship and abundance information between individual sample sequences, and to explore whether there were significant microbial community differences between groups by calculating the distance between them. We visualized the data and used the Bray-Curtis distance matrix heat map and the Unweighted-Unifrac distance matrix PCoA map to distinguish the patient group from the control group. In addition, LEfSe statistical analysis (LDA) was used to test the significance of difference in species composition and community results of the grouped samples.

RESULTS

Characteristics of sequencing data
Overall, the average OTUs of these 23 samples was 1,050, with a maximum of 1,999 and a minimum of 285. The overall statistics information of OTUs was summarized in Table 1. The OTUs of each independent sample was shown in Figure 1.

Shannon dilution curve was mainly used to further evaluate whether the sequencing volume was sufficient and indirectly reflects the richness of species in these stool samples. When the curve was flattened or reached a plateau, it indicated that the sequencing depth was enough, otherwise the growth of OTUs with continuous sequencing will affect the following diversity analysis. Based on this, our experimental results showed that the Shannon dilution curve between groups reflected sufficient sequencing depth and basically covered all species (Figure 2).

The differences in intestinal microecology between the two groups
To understand whether there is a significant difference in microbial community between the newly diagnosed patient group and healthy control group, we used the evolutionary relationship and abundance information between individual sample sequences to calculate the distance between samples. According to the Bray-Curtis distance matrix heat map (Figure 3), the distance in group A was relatively close, and so was the distance in group B. However, the distance between groups A and B was relatively far from each other, so it implied that there was a difference between them.

Consistently, the PCoA diagram of the Unweighted Unifrac distance matrix exhibited a more significant difference (Figure 4). PCoA considered the evolutionary relationship and combined similar clustering species in the evolutionary structures. The dimensionality reduction analysis shows that the first principal coordinate (PCO1) with the largest contribution rate is 12.4% and the second principal coordinate (PCO2) with the largest contribution rate is 7.5%. The samples with highly similar community structures tended to gather together, while the samples with large differences in community were far away from each other. Our data demonstrated that the distance within group A or group B was relatively short, whereas the distance between the two groups was relatively far, indicating that the disease harbors its unique microecological structure.

Characteristics of intestinal colony structure in samples
To reveal the distinct microbial structures in the two groups of samples, the results of the experiment were further annotated using an RDP comparison annotation software. Three levels of phylum, genus, and species were selected for comparative analysis. As shown in Figure 5, differences in species and genera were observed in microbial composition between different independent samples. Briefly, at the phylum level, Bacteroidetes and Firmicutes accounted for the main abundance in both groups. Compared with healthy controls (group B), the abundance of Bacteroidetes in the phyla of group A was lower than that of group B, while Firmicutes in group A was higher than that of group B. Accordingly, at the species level, *Bacteroides fragilis* and *Bacteroides ovatus* were less abundant in group A. This phenomenon suggests that certain strains of Bacteroidetes may be reduced due to hematological malignancies, while other strains of this type are likely to increase in this study. Its underlying mechanism remains to be clarified.

Specific intestinal flora in patients with hematologic malignancies
LEfSe analysis was performed to screen OTUs of microorganisms with substantial differences (Figure 6). In this approach, Kruskal-Wallis rank sum test was used to detect species with significant abundance differences in large classifications, and then Wilcoxon rank sum test was selected to further detect species with significant abundance differences in small classifications (subspecies). Finally, linear discriminant analysis (LDA) was chosen to estimate the impacts of landmark species identified in the previous two steps.

According to LDA scores from phylum to species in the healthy control population (group B), the LDA value of Bacteroides was relatively high, which could likely be considered as a marker of the healthy population.

Table 1. Sample population OTUs statistics.

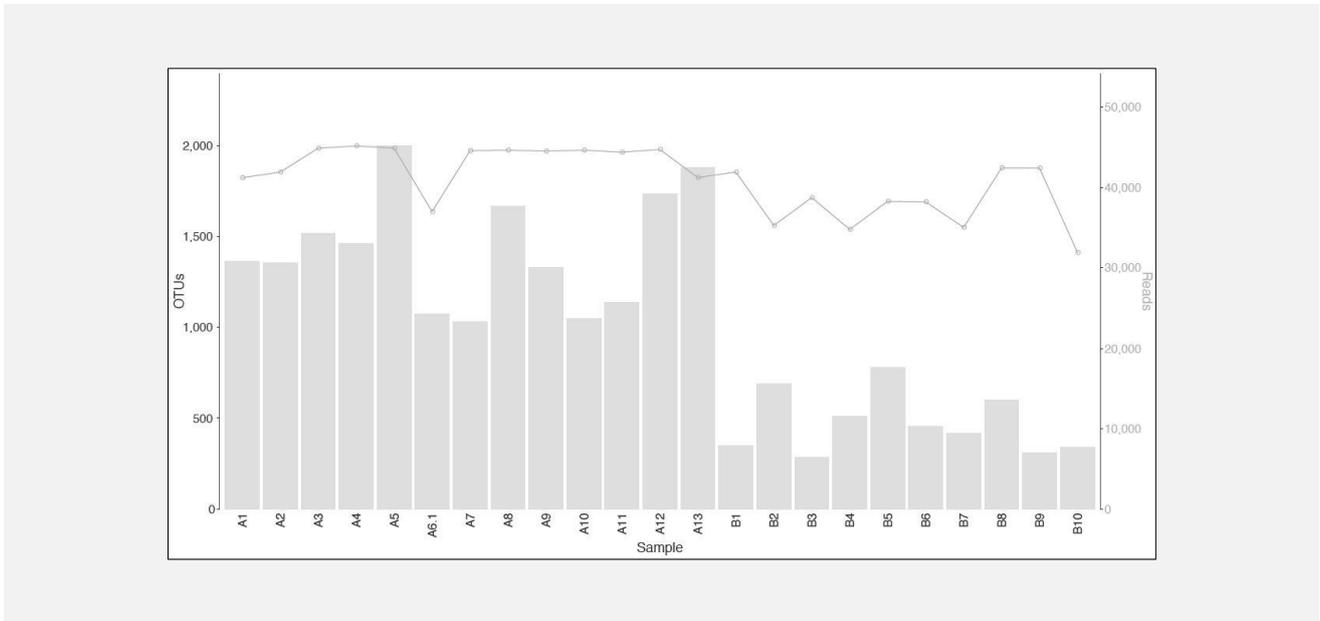| Sample	Min	Max	Median	Mean	Std. dev
OTUs	285	1,999	1,050	1,014.65	114.79
Reads	31,905	45,194	41,938	41,007	840.37

Figure 1. All OTU statistics.

The grey bar chart showed OTU information, and the grey line chart showed the number of reads.
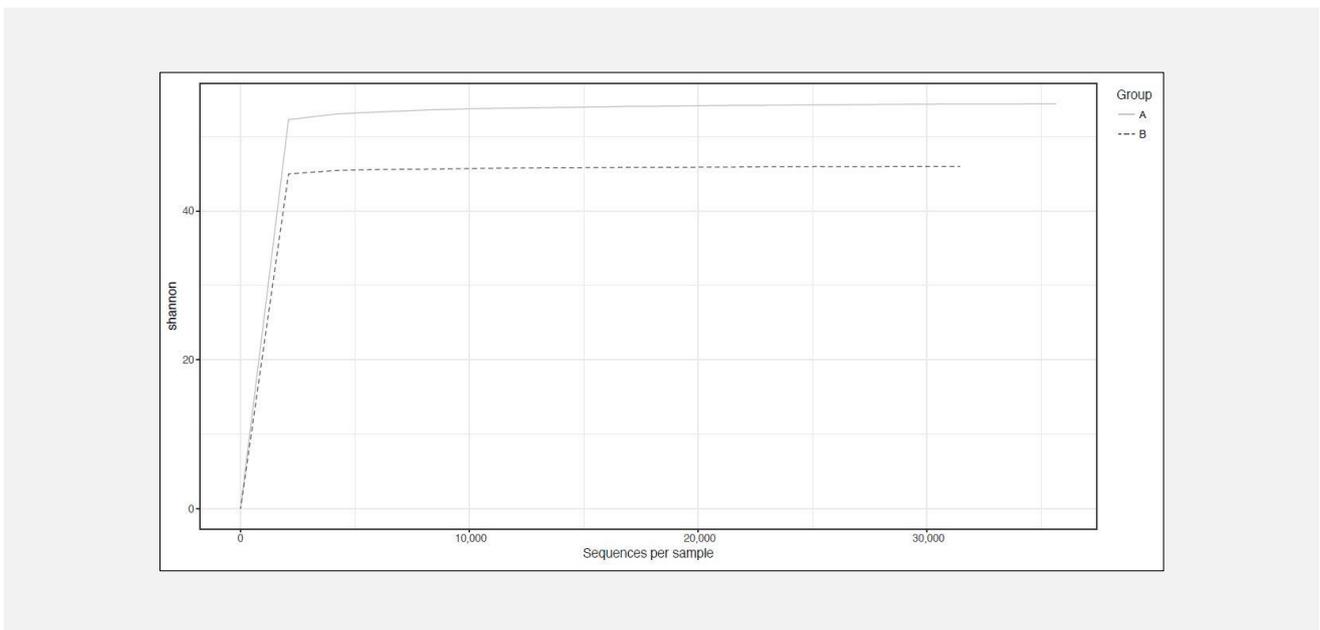

Figure 2. Shannon dilution curve between groups.

Solid curve indicates group A (Patients), and dashed curve indicates group B (Controls).
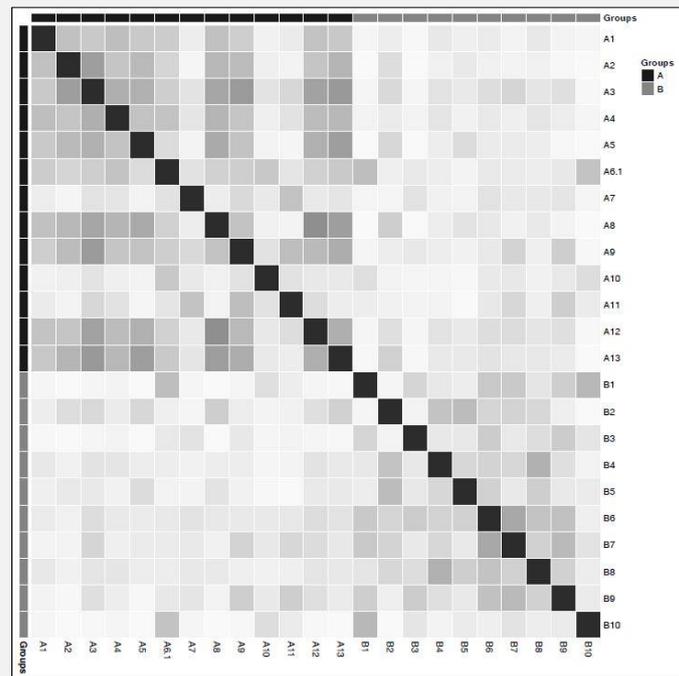
Figure 3. Matrix heat map based on the Bray-Curtis distance of all samples.

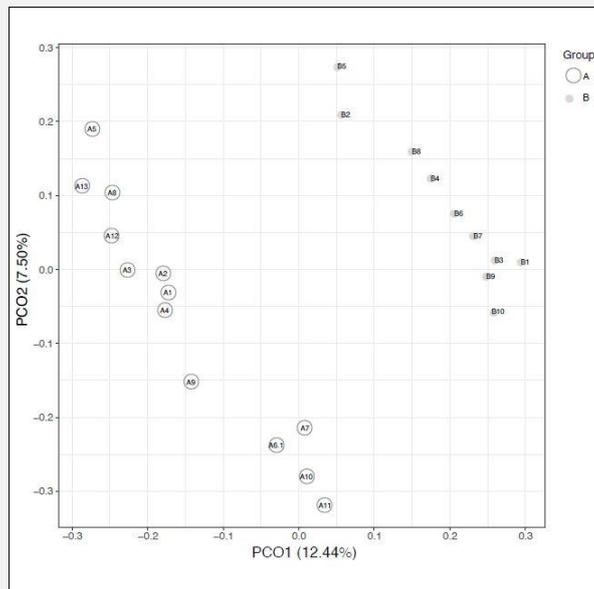**The deeper the color, the shorter distance between samples.**

Figure 4. PCoA of 23 stool samples based on Unweighted-Unifrac distance.

White circle indicates group A (Patients), and grey circle indicates group B (Controls).

Figure 5. The relative abundance of the annotated OTUs for all samples (a. phylum b. genus c. species).
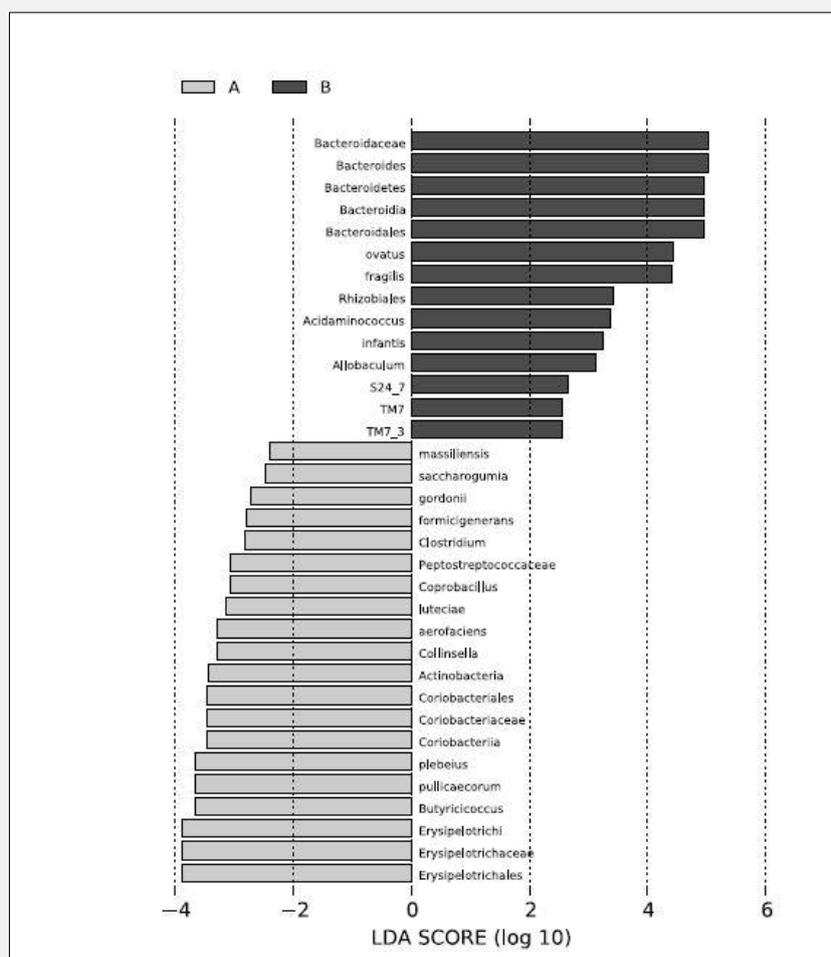
Figure 6. The LDA values of microbes with significant abundance differences in samples.

Grey bar represents stool samples from patients with hematologic malignancies, black bar represents stool samples from healthy controls.

Erysipelotrichi, Erysipelotrichales, and *Erysipelotrichaceae* could be regarded as markers for patients with hematological malignant diseases. *Butyricicoccus pullicaecorum*, *Bacteroides plebeius*, and *Collinsella aerofaciens* were also determined to gain higher LDA scores, implying their potential value as markers in patients with hematological malignancies.

DISSCUSION

Characteristic flora in intestinal microecological structure of healthy individuals
The findings of the present study unveiled that Bacteroidetes, Bacteroidia, Bacteroidales, and *Bacteroides fragilis* could likely be used as potential biomarkers for healthy people, suggesting their important role in healthy intestinal flora, which was consistent with a series of previous studies [13-15]. Bacteroidetes play a role in metabolic diseases such as obesity [16]. However, how they interact with host and their internal mechanisms require further research to clarify. A recent study suggests that fecal IgA levels are determined by strain-level differences in *Bacteroides ovatus*. As the first immune line of intestinal mucosal barrier, IgA plays an important role in health. In our study, characteristics of this bacterium were also found in intestinal flora of healthy control group [17]. The results of this study also verified the intestinal microbial community structure of healthy adults and children reported by Panagiotis Agioutsantis et al. and found the value of *Bacteroides fragilis* in healthy adults [18].

Characteristic flora in intestinal microecological structure of patients with hematological malignancies

Erysipelotrichales, Erysipelotrichales, and *Erysipelotrichaceae* identified in this study were significantly increased in the intestinal flora of patients with hematological malignancies, which was consistent with the report by Duy MD et al. [19]. They found that the relative abundance of Erysipelotrichales, Erysipelotrichales, and *Erysipelotrichaceae* increased significantly in patients with HIV, and the increase of the bacteria was positively correlated with the elevation of IL-1β. IL-1β has been shown to affect the transcriptional regulation of human breast cancer cells, and its elevation can augment tumor susceptibility and enhance tumor invasiveness [20]. Erysipelotrichales was also observed in the intestinal flora of patients with esophageal squamous cell carcinoma (ESCC) compared with healthy subjects or patients with esophagitis [21]. Clarissa Schwab et al. described changes in intestinal flora in mouse models of acute inflammatory reactions, suggesting that Erysipelotrichales with low abundance increased during this process, which was in parallel with the increase in inflammatory markers in the host [22]. From these previous studies, it may be concluded that Erysipelotrichales increases with original relatively low abundance in the course of inflammatory responses, and cancer occurrence and progression. Patients with hematopoietic tumors and malignant hematopoietic stem cell defects usually manifest low immunity, and susceptibility to infection leads to an inflammatory response, which has been confirmed by the aforementioned phenomenon.

Bacteroides plebeius is a Gram-negative anaerobe. Nevertheless, its role in the course of disease is rarely studied. The abundance of *Bacteroides* in the vaginal flora of women infected with human papillomavirus was found to be relatively high [23]. The study of Giorgia Mori showed that *Bacteroides plebeius* was spotted in a series of bacterial communities in the intestinal microecology of patients with Lynch syndrome (LS) that might be associated with cancer genes [24]. LS is one of the common genetic cancer syndromes, which is prone to cancer and has a lifetime risk to inflict colorectal cancer (CRC), endometrial cancer (EC), ovarian cancer (OC), and some other tumors. In our study, the presence of *Bacteroides plebeius* was also observed in the intestinal microecological composition of patients with hematological malignancies. Hence, these results raise an interesting question whether *Bacteroides plebeius* is associated with a range of cancers.

Butyricicoccus pullicaecorum is a butyrate producing anaerobe belonging to the genus clostridium, which has been shown to have probiotic action in a series of studies. It exerts a significant protective effect on IBD patients by producing butyrate, reducing MPO, TNF-α and IL-12 [25]. *Butyricicoccus pullicaecorum* supernatant prevented the loss of TNF-α and IFN-γ induced transepithelial resistance (TER) and increased IL-8 secretion in the CACO-2 cell model [26,27]. IBD patient's symptoms were improved with the presence of *Butyricicoccus pullicaecorum*, while its role in patients with hematological malignancy remains unclear [28].

Collinsella aerofaciens is a Gram-positive actinomycete commonly located in human gut flora. Kalin KA et al. found in the study of inflammatory joint diseases that *Collinsella aerofaciens* was involved in the formation of pre-inflammatory immune state, which increased intestinal permeability by reducing the expression of tight junction proteins and generating collagen destruction products in IEC. Subsequently, it could also induce the expression of IL-17, the chemokines CXCL1 and CXCL5, indicating that this bacterium can reduce the integrity of intestinal barrier in the intestinal inflammatory environment, leading to the occurrence and maintenance of inflammatory diseases [29]. In patients with malignant melanoma treated with anti-PD-1 immunotherapy, *Collinsella aerofaciens* was determined to be more abundant in the intestinal flora of patients with effective response to the immunotherapy compared with patients without response. Moreover, the reconstruction of germ-free mice with bacterial colony structures from responsive patients was able to enhance T cell response and improve the efficacy of immunotherapy. This leads us to speculate whether the role of *Collinsella aerofaciens* in the development of hematological malignancies is related to the prognosis of therapeutic efficacy, suggesting a new trial design is needed to further explore this issue.

Advantages and Limitations

In the experimental design phase of this study, the interference factors such as antibiotics, hypertension, and diabetes that have an impact on intestinal microecology were eliminated. We collected samples before standardized management of patients with malignant blood diseases, which is helpful to reduce the deviation of results caused by interference factors such as antibiotics, chemotherapy drugs, and surgery, and improve the scientific nature and reliability of conclusions. In terms of analysis methods, this study understood the basic situation of the data through OTU cluster analysis. Alpha diversity was used to explore the abundance and uniformity within a single sample, and Beta diversity was used to explore whether there were differences among samples. In this process, multiple algorithms were used to verify the distance at the same time to ensure the accuracy of clustering. According to a series of previous studies, we can conclude that the interventions to reverse gut dysbiosis and regulate inhibitory immune re-activation could be new strategies for immune reconstruction in patients with malignant diseases, which may also be a hope to improve the prognosis of hematological malignancy patients in the future.

As many patients with malignant blood diseases have fever symptoms, antibiotics are often used empirically in clinical treatment without definite diagnosis. It has been recognized that antibiotics affect the intestinal microecology, and some common chronic diseases are

known to affect the intestinal flora. Due to the strict sample screening conditions, only 13 qualified samples were finally collected. The recent study of intestinal flora in multiple myeloma patients, published in the journal Microbiome, also collected only 19 eligible samples, indicating that studies on malignant blood diseases are difficult to collect on a large scale [30]. Future experiments need to classify hematological malignancies into acute lymphocytic leukemia, acute non-gonococcal leukemia, myelodysplastic syndrome, and other implications to study the specific structures of intestinal flora of these diseases in more detail. Besides, despite the results, the present study illustrated that differences existed in terms of the intestinal microecology between hematological malignant subjects and healthy individuals, and a series of molecular mechanisms, such as how the different bacteria interact with the host, need to be further explored.

CONCLUSION

In this study, we investigated the intestinal microecological structure of 13 newly diagnosed patients with hematological malignancies and 10 healthy individuals from the First Affiliated Hospital of Anhui Medical University, China. All the samples were used to undergo 16s rDNA high-throughput sequencing. The results indicate that Erysipelotrichi, Erysipelotrichales, and *Erysipelotrichaceae* can likely be considered as markers of malignant diseases of the blood system. *Butyricicoccus pullicaecorum*, *Bacteroides plebeius*, and *Collinsella aerofaciens* also shed light on potential values as markers of intestinal flora for patients with hematological malignant diseases. Compared with other studies, the findings of this research were consistent with those on intestinal flora of malignant tumors, and some special flora with less relevant studies were also found such as *Bacteroides plebeius*, *Butyricicoccus pullicaecorum*, and *Collinsella aerofaciens*, which suggest a new direction for the future research. Therefore, it is necessary to further study the molecular mechanisms of the action of specific bacteria on diseases.

Declaration of Interest:
The authors declare that they have no competing interest.

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