Changes in the intestinal microbiota of multiple myeloma patients living in high-altitude and cold regions analyzed using 16s rRNA high-throughput sequencing

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Abstract. Multiple myeloma (MM) is a plasma cell clonal disease and these plasma cells can survive in the gut. The intestinal microbiota is a complex ecosystem and its dysfunction can release persistent stimulus signals that trigger genetic mutations and clonal evolution in the gut. The present study analyzed the intestinal microbiota in fecal samples of MM patients in high-altitude and cold regions of China using 16s rRNA sequencing and analyzed significantly enriched species at the phylum and genus levels. Although no significant difference in the alpha diversity was observed between the MM and control groups, a significant difference was noted in the beta diversity. A total of 15 significant differential bacteria at the genus level were found between the two groups, among which *Bacteroides*, *Streptococcus*, *Lactobacillus* and *Alistipes* were significantly enriched in the MM group. The present study also constructed a disease diagnosis model using Random Forest analysis and verified its accuracy using receiver operating characteristic analysis. In addition, using correlation analysis, it demonstrated that the composition of the intestinal microbiota in patients with MM was associated with complement levels. Notably, the present study predicted that the signaling and metabolic pathways of the intestinal microbiota affected MM progression through Kyoto Encyclopedia of Genes and Genomes functional analysis. The present study provides a new approach for the prevention and treatment of MM, in which the intestinal microbiota may become a novel therapeutic target for MM.

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Introduction

Multiple myeloma (MM) is a malignant hematological disease characterized by abnormal proliferation of clonal plasma cells in the bone marrow, accompanied by the secretion of large amounts of ineffective monoclonal immunoglobulins in the serum or urine (1). This abnormal proliferation ultimately leads to specific end-organ damage, including hypercalcemia, renal insufficiency, anemia and osteolytic lesions (2). There are ~140,000 new cases of MM are reported globally annually, with an increasing trend per year (3). In most countries, MM is the second most common malignant tumor of the blood system and epidemiological studies have shown that it is distributed regionally (4). In the past decade, with the emergence of new chemotherapy drugs and development of high-level antitumor regimens, the prognosis of patients with MM has greatly improved (5). However, almost all patients will eventually relapse, even those with a complete response to initial treatment (6).

The intestinal microbiota is a complex ecosystem composed of thousands of microorganisms that participate in nutrient metabolism and absorption in the host gut, as well as regulating host intestinal immunity through the mesenteric lymphatic system (7). The immune regulatory effect of the intestinal microbiota extends beyond the gut, primarily through small molecules they produce (8). The anaerobic environment in the gastrointestinal tract enables bacteria to ferment and produce metabolites with immune activity that can enter the systemic circulation, especially butyrate produced by *Clostridium butyricum* (9). Disturbance of the intestinal microbiota and an abnormal increase in its metabolites can lead to continuous stimulation signals throughout the entire gastrointestinal tract and trigger various diseases, including tumors (10). Previous studies have evaluated the relationship between intestinal microbiota and other hematological tumors (11); however, MM has rarely been studied, especially in high-altitude and cold regions.

Antibody-secreting plasma cells can survive in the gut for a long time (12). The composition of the intestinal microbiota can influence the degree of antigen stimulation in these cells and may play a role in the development of mutations and

clonal evolution. Drugs used to treat MM, such as proteasome inhibitors and alkylating agents, often cause adverse gastrointestinal reactions (13). Several studies have shown changes in the composition and abundance of intestinal microbiota in MM patients undergoing hematopoietic stem cell transplantation (HSCT) (14-16). The present study hypothesized that certain intestinal microorganisms and their metabolites play regulatory roles in the progression of MM. The present study performed 16s rRNA high-throughput sequencing to characterize the intestinal microbiota of patients with MM and found changes in the abundance of several bacteria. It also conducted correlation and Kyoto Encyclopedia of Genes and Genomes (KEGG) function prediction analyses. The present study provided a theoretical basis for elucidating the pathogenesis of MM and a new approach for its prevention and treatment.

Materials and methods

Patients. A total of 15 newly-diagnosed patients with MM, who met the diagnostic criteria of the International Myeloma Working Group (IMWG) (17), were enrolled at the first hospital of Qiqihar between October 2021 and March 2023. All patients originated from the northwest of Heilongjiang Province and the Inner Mongolia Autonomous Region, which are high-altitude and cold regions of China. A total of 11 healthy individuals from the spouses, children and parents of the patients were recruited as controls, because they shared a common living environment and dietary habits similar to those of the patients. Patients with gastrointestinal diseases or diarrhea, infectious diseases, or metabolic disorders; or those who had recently taken antibiotics, gastrointestinal motility drugs, microecological regulators, or immunosuppressants were excluded from the study. No significant differences in age, sex and basal metabolic rate were observed among the enrolled subjects (Table I).

Sample collection. Fecal samples were collected from patients before systemic treatment and from healthy controls in the early morning. To avoid sample contamination, the patients were instructed to defecate in a clean container. An appropriate sample was collected using a disposable sample spoon and placed in a sterile closed container. To avoid interference from environmental factors during the experiment, the entire sampling process did not exceed 10 min. Samples were stored at -80˚C until transport to the testing laboratory with dry ice. Venous blood (5 ml) was collected from patients and healthy volunteers on an empty stomach and centrifuged at 2,000 x g and room temperature for 10 min. Serum was then collected and was stored at -20˚C until further detection of complement components, such as C1q, C3 and C4 (BN II; Siemens AG).

Sample DNA extraction. Total genomic DNA in fecal samples from the two groups was extracted using the PF Mag Bind Stool DNA Kit (Omega Bio-Tek, Inc.) according to the manufacturer's instructions. The integrity of the extracted genomic DNA was detected by 1% agarose gel electrophoresis. The concentration of the DNA was determined using NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Inc.).

16s rRNA sequencing and paired-end (PE) library construction. Using the extracted DNA as a template, the V3-V4 hypervariable region of the 16s rRNA gene was amplified with primer pairs 338F (5'-ACTCCTACGGGGGGGGCAG-3') and 806R (5'-GACTACHVGGGTWTCTAAT-3') using the ABI GeneAmp 7500 PCR thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). Amplicons were recovered using 2% agarose gel, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences; Corning, Inc.) and quantified using a Quantus Fluorometer (Promega Corporation). A PE library was constructed using the NEXTFLEX Rapid DNA-Seq Kit (BioScientific, Inc.) and PE sequencing was performed on an Illumina MiSeq PE300 platform (Illumina, Inc.). The raw sequencing data were uploaded to the Majorbio Cloud platform (https://cloud.majorbio.com; Majorbio Bio-Pharm Technology Co. Ltd) for bioinformatics analysis.

Bioinformatics analysis. Raw sequencing data were subjected to quality control and splicing using the Fastp software (https://github.com/OpenGene/fastp, version 0.19.6) and Flash software (https://ccb.jhu.edu/software/FLASH/index.shtml, version 1.2.11), respectively. The optimized sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity using the UPARSE software (http://www.drive5.com/uparse/, version 11). Sequences annotated as chloroplasts and mitochondria were removed from all samples and the number of 16s rRNA gene sequences in all samples was rarefied to 20,000. OTU taxonomic annotation was performed based on the Silva 16s rRNA gene database (https://www.arb-silva.de/, version 138) using the RDP classifier software (https://sourceforge.net/projects/rdp-classifier/, version 11.5). Functional prediction analysis was performed using PICRUSt2 (http://picrust.github.io/picrust/, version 2.2.0). All bioinformatic analyses were conducted using the Majorbio Cloud platform (https://cloud.majorbio.com; Majorbio Bio-Pharm Technology Co. Ltd).

Statistics analysis. The comparison of age and BMI among the general characteristics of the subjects was conducted by t-test and the sex by chi-square test. The ACE, Chao, Sobs and Shannon indices were used to evaluate alpha diversity and the Wilcoxon rank-sum test was used to analyze intergroup differences. Principal coordinate analysis (PCoA) based on the Bray-Curtis distance algorithm was used for beta diversity analysis and analysis of similarities (ANOSIM) was used to determine statistical significance. Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify bacterial groups with significant differences (LDA>2; $P<0.05$) in abundance from phylum to genus levels. Disease diagnosis model constructed by Random Forest (RF) analysis, and ROC curve analysis was used to verify the accuracy of the constructed model. The Mantel test and distance-based redundancy analysis (db-RDA) were used for environmental factor correlation analysis to investigate the effects of clinical indicators on the composition of the intestinal microbiota. Network analysis was performed using Spearman correlation ($\varrho>0.6$; $P<0.05$). PICRUSt2 was used to predict KEGG functional genes. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. General characteristics of the subjects.

Characteristic	MM (n=15)	Healthy control (n=11)	P-value
Age, years	66.13±1.89	62.09±2.36	0.189
Sex (Male/Female)	11/4	7/4	0.683
BMI, kg/m^2	26.87±0.93	25.74±1.56	0.446
R-ISS Stage	I (3) II (6) III (6)		
Disease Subtype	IgG-κ (5) IgG-λ (3) IgM-κ (1) Light chain-λ (4) Unknown (2)		

Results

Microbial diversity analysis. The present study obtained taxonomic information on the microbiota through OTU analysis and evaluated species abundance and distribution uniformity using rank-abundance curves. Analysis of the rank-abundance curve showed that species richness and evenness in the MM group were higher compared with the healthy group; however, the proportion of dominant bacteria in the MM group was lower (Fig. 1A). Pan analysis showed that, as the number of samples increased, the total number of fecal species increased, especially in the MM group, indicating that the sample size in this study was relatively small (Fig. 1B). However, as the sample size increased, the decrease in shared OTU tended to plateau through core analysis, indicating that the sample size of this experiment was acceptable (Fig. 1C). The Ace, Chao, Sobs and Shannon indices were calculated to evaluate the alpha diversity in the two groups. The Wilcoxon rank-sum test showed that alpha diversity in the MM group was higher compared with that in the control group, but the difference was not significant (Fig. 1D). Dilution curve analysis, including Sobs and Shannon, showed that the curve reached a plateau, indicating that this sequencing covered almost all the bacteria in the sample (Fig. 1E and F), thus ensuring the rationality of subsequent analysis.

Microbial composition analysis. The representative sequences of the OTUs were compared with the microbial reference database to obtain species information. To facilitate the search for microbial disease markers, a Venn analysis was performed to count the number of species (such as OTUs) that were common and unique to the MM and control groups. The results showed that 439 OTUs were shared between groups and 389 OTUs were unique to MM (Fig. 2B). To understand the composition of the microbial communities, those significantly enriched were displayed through community bar plot analysis (Fig. 2A). At the phylum level, Firmicutes, Proteobacteria, Bacteroides, Actinobacteria, Verrucomicrobiota, Fusobacterota, Desulfobacterota, Patescibacteria and Synergistota were abundant in both groups. Furthermore, pie plots were used to show the proportion of the dominant species in the microbial community (Fig. 2D). Community heatmap analysis was used to display significantly enriched taxa at the genus level, with Escherichia-Shigella, Blautia, Bacteroides, Bifidobacterium and Streptococcus the most abundant (Fig. 2C). Next, significant group-specific species were searched for.

Differential species analysis. The present study performed beta diversity analysis using PCoA to evaluate the differences between the MM and control groups (Fig. 3A). PCoA is a non-binding dimensionality reduction analysis method that can be used to study similarities or differences in the composition of sample communities. Each sample is represented as one point and the closer the two points are, the more similar the species composition. Moreover, the present study conducted an ANOSIM on the grouped samples to test the significance of the differences between the two groups. ANOSIM analysis showed a significant difference in the microbial composition between the MM and control groups ($R=0.1446$; $P<0.05$; Fig. 3C). Intergroup differences at the genus level were then analyzed. LEfSe analysis revealed significant differential bacteria from the phylum to genus levels between the two groups (Fig. 3D). Furthermore, bacterial genera with significant differences between the MM and control groups were specifically identified at the genus level using the Wilcoxon rank-sum test. A total of 15 bacterial genera were significantly different between the two groups, among which Bacteroides, Streptococcus, Lactobacillus and Alistipes were significantly enriched in the MM group (Fig. 3B).

Correlation and functional prediction analyses. To investigate the role of different species in disease diagnosis, Random Forest analysis was used to construct a disease diagnostic model (Fig. 4A) and then receiver operating characteristic (ROC) analysis was used to verify the accuracy of the constructed model (Fig. 4B). The results showed an area under curve (AUC) of 0.88 [95% confidence interval (CI): 0.72-1.00], indicating that this diagnostic model was accurate. To understand the effect of clinical indicators on the intestinal microbiota, The Mantel test was used to verify

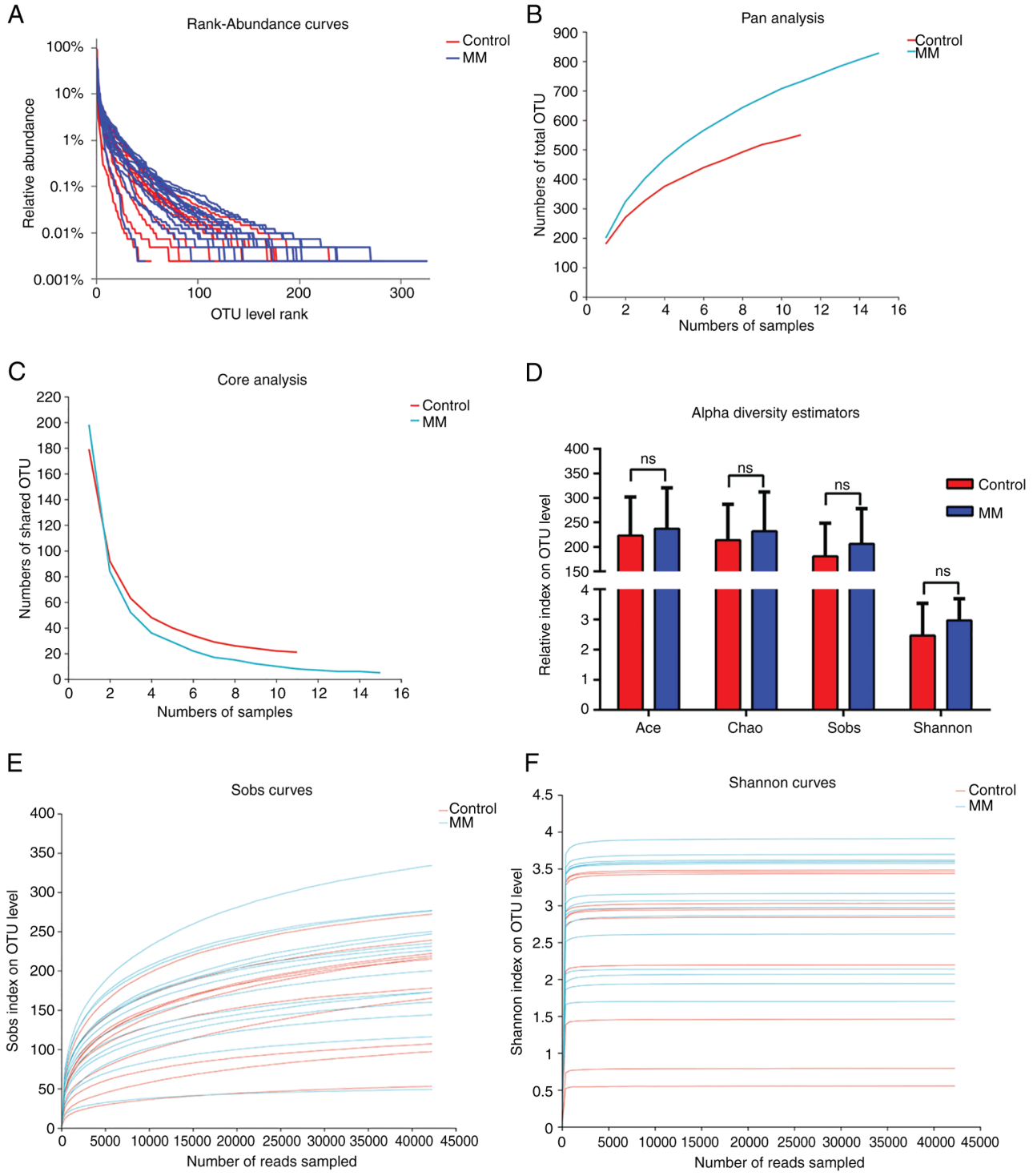

Figure 1. Alpha diversity analysis of the intestinal microbiota between the MM and control groups. (A) Rank-abundance curve analysis showing species richness and evenness. (B) Pan analysis evaluating sample size. (C) Core analysis evaluating core species uniformity. (D) Ace, Chao, Sobs and Shannon indices. (E) Sobs dilution curve evaluating sequencing quantity. (F) Shannon dilution curve evaluating sequencing quantity. MM, multiple myeloma; ns, no significant differences; OUT, operational taxonomic unit.

the correlation between the community distance matrix and the complement system. Correlation analysis showed that the top eight differential species were related to the content of serum complement C1q, while a significant correlation was observed between C3 and C4, two clinical indicators (Fig. 4C). Additionally, db-RDA analysis showed a positive correlation between the levels of complement C3 and C4 and intestinal microbiota species composition in the MM group and a negative correlation with C1q, while the control group showed the opposite result (Fig. 4E).

Single-factor correlation network analysis was performed to determine the interactions between the species in the sample. The 50 most abundant species were selected at the genus level and the Spearman correlation coefficient (ϱ) was calculated to examine correlations among the species ($r>0.6$; $P<0.05$). The size of the nodes represents species abundance, the red line

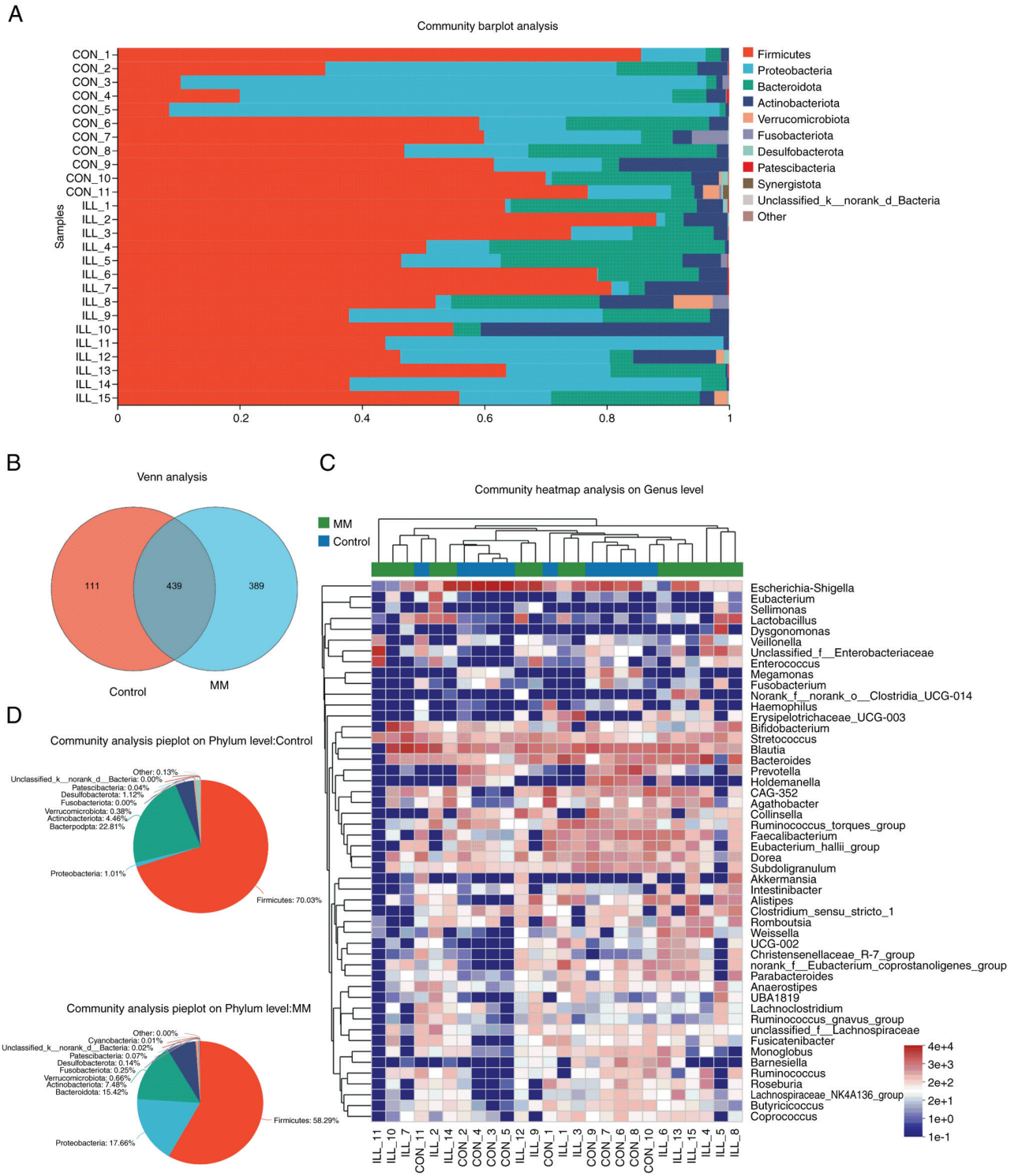

Figure 2. Microbial composition analysis of the MM and control groups. (A) Community bar plot analysis at the phylum level. (B) Venn analysis showing species shared and unique to the MM and control groups. (C) Community heatmap analysis on genus level. (D) Pie plots showing the proportions of dominant species in the community. MM, multiple myeloma.

represents a positive correlation, the green line represents a negative correlation and the thickness of the line represents the size of the correlation coefficient (Fig. 4D). the KEGG database was used to predict and compare the functional genes related to metabolic pathways of microbial communities in different sample groups. Compared with the control group, gene expression involved in *Staphylococcus aureus* infection, apoptosis, central carbon metabolism in cancer and glycolysis/gluconeogenesis were significantly increased in the MM group, while those in choline metabolism in cancer and C5 branched basic acid metabolism were significantly reduced. The KEGG function prediction was performed using PICRUSt2 software package that is based on 16S amplicon sequencing results. Unlike metagenomic sequencing, KEGG function prediction using PICRUSt2 does not have gene count thresholds (Fig. 4F).

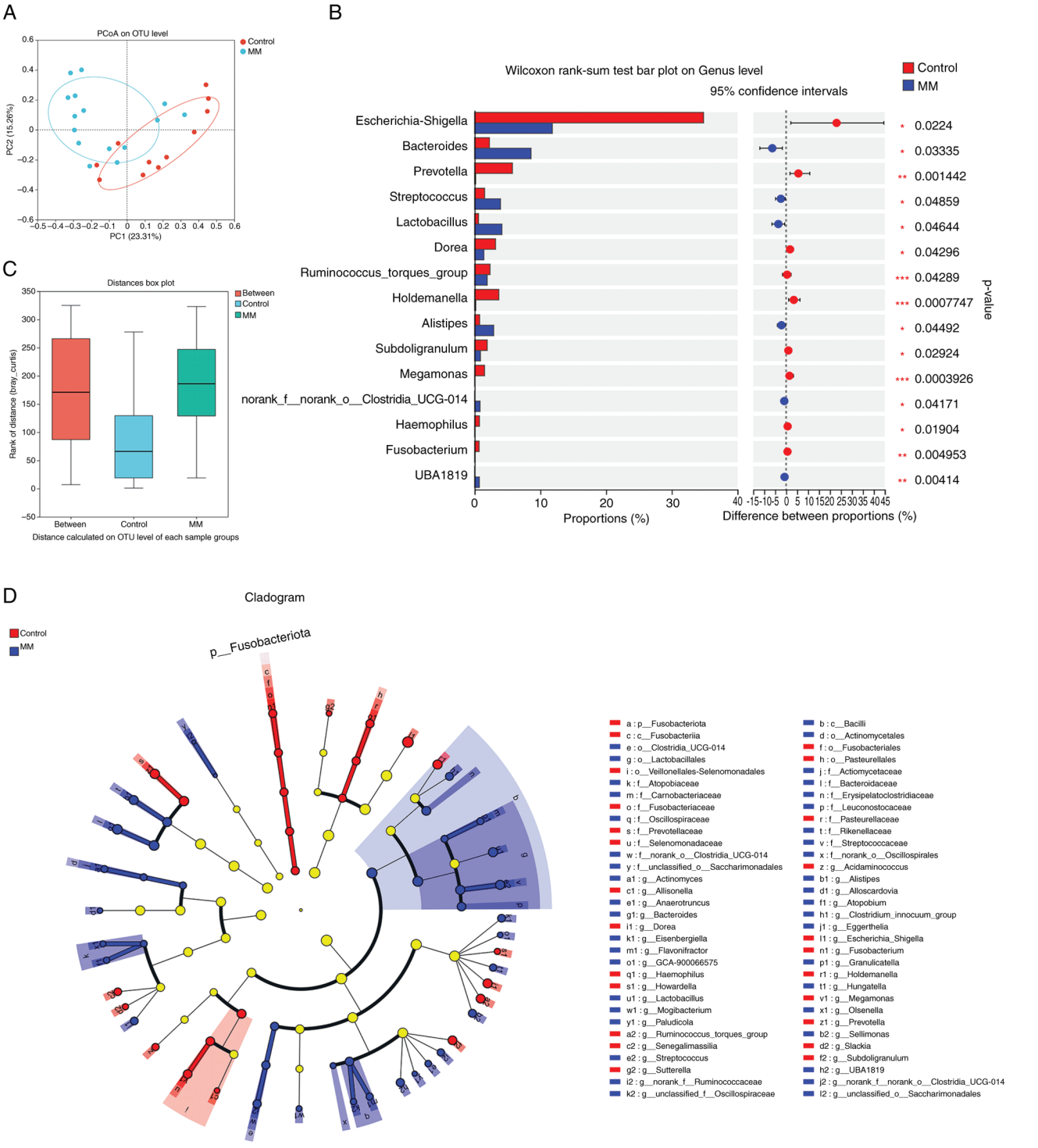

Figure 3. Differential species analysis between the MM and control groups. (A) Beta diversity analysis using PCoA. (B) Differential species at the genus level using the Wilcoxon rank-sum test, P<0.05, significant differences. (C) Analysis of intergroup differences using ANOSIM. (D) Species differential from the phylum to genus levels using LEfSe analysis, LDA>2.0 and P<0.05. MM, multiple myeloma; PCoA, principal coordinate analysis; ANOSIM, analysis of similarities; LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis.

Discussion

Plasma cells are major participants in adaptive immunity owing to their ability to produce immunoglobulins and resist microbial infections (18). When plasma cells mutate into clones, abnormal globulins (monoclonal proteins) are produced and these immunogenic proteins can drive the immune system to resist the host (19). Researchers from the University of Oslo in Norway found that plasma cells, especially those lacking CD19 and CD45 expression, can survive in the gut for decades (12). The intestinal microenvironment is rich in immune stimuli from normal flora and external pathogens and long-term exposure to these antigens increases the chance of genetic mutations that can cause clonal plasma cell generation and plasma cell tumors such as multiple myeloma (20). The present study indicated that the

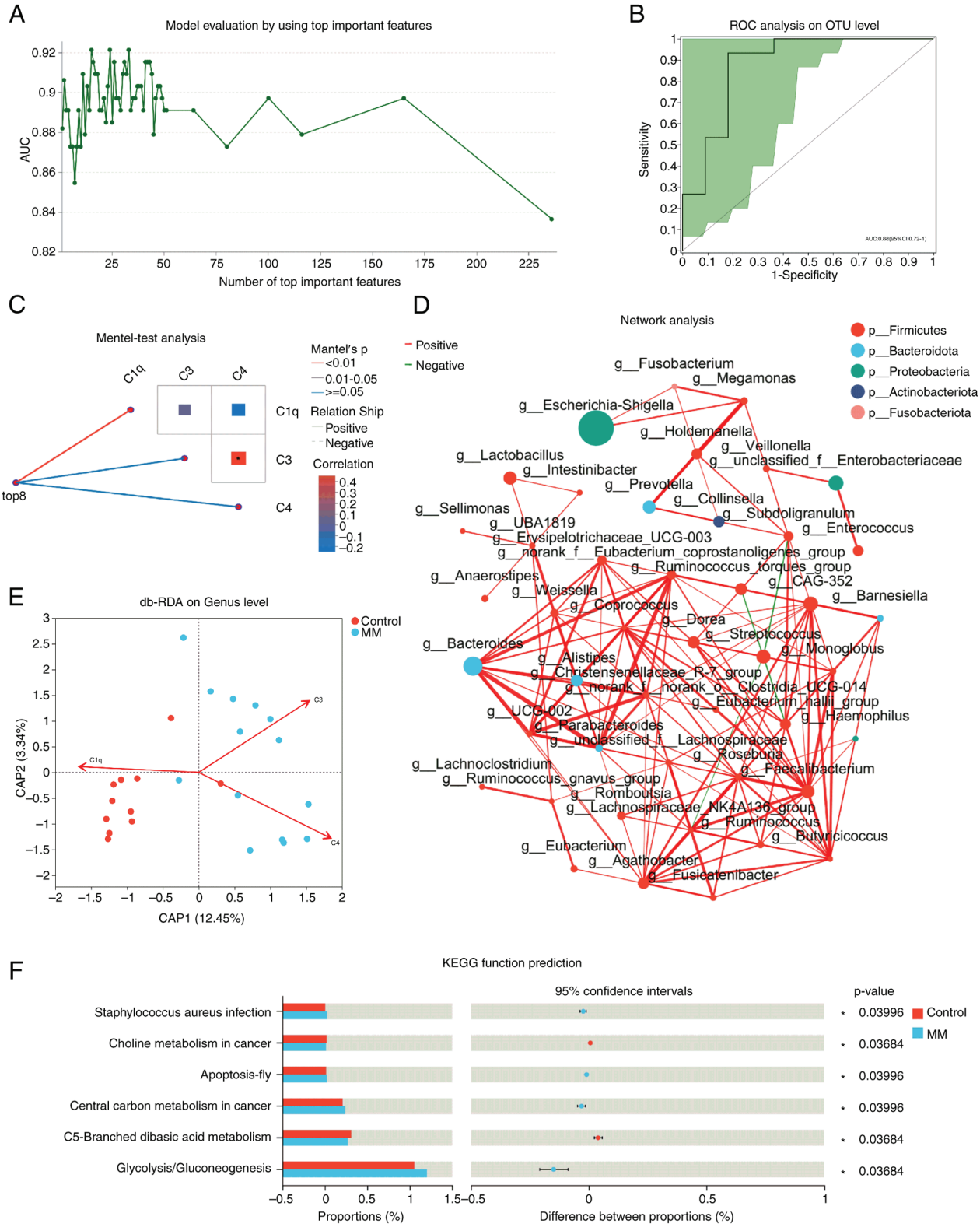

Figure 4. Correlation and functional prediction analyses of the MM and control groups. (A) Disease diagnosis model constructed using RF analysis. (B) ROC curve analysis was used to verify the accuracy of the constructed model (AUC: 0.88, 95% CI: 0.72-1.00). (C and E) Mantel test and db-RDA analysis showing the correlation between serum complement components (C1q, C3 and C4) and species composition. (D) Network analysis showing the interactions between species in the samples. (F) Differential function prediction analysis of KEGG metabolic pathways. MM, multiple myeloma; RF, Random Forest; ROC, receiver operating characteristic; AUC, area under curve; CI, confidence interval; db-RDA, distance-based redundancy analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.

occurrence of MM may be related to changes in the intestinal microbiota and multiple correlation and prediction analyses were conducted.

Monoclonal gammopathy of undetermined significance (MGUS) is a precursor disease of MM (21). Pepeljugoski *et al* (22) performed the first intestinal microbiota

detection in patients with MGUS and MM. Compared with healthy controls, *Odoribacter* and *Lactobacillus* were the most enriched genera in patients with MM, whereas *Blautia* and *Faecalibacterium* were the most reduced genera. Compared with the MGUS group, *Kluyvera* and *Bacteroides* had the highest abundance, whereas *Blautia* and *Parabacteroides* had the lowest abundance in the MM group. Moreover, the microbial diversity in patients with MM and MGUS was higher than that in healthy individuals. Their study confirmed the distinct differences between MM and its precursor disease in the intestinal microbiota at the genus level, suggesting that an imbalance in the intestinal microbiome may be related to MM progression. However, a study by Zhang *et al* (23) showed that the Shannon index in patients with MM was lower compared with that in healthy patients, indicating that the diversity of the intestinal microflora in patients decreased. Compared with healthy controls, the proportions of *Bacteroides*, *Faecalibacterium* and *Roseburia* in MM increased at the genus level. Similarly, the present study showed that *Bacteroides* was the most abundant species in MM. Compared with patients with MM in plain areas, *Streptococcus*, *Lactobacillus and Streptomyces* were also significantly more abundant, but an increase in the proportion of *Faecalibacterium* and R*oseburia* was not observed. In addition, the alpha diversity of the intestinal microbiota in MM patients living in high-altitude areas was higher compared with that in the healthy control group, but the difference was not significant. Random Forest analysis was also used to select important microorganisms (biomarkers) to construct a disease diagnostic model and validated the accuracy of the constructed model using ROC analysis. The AUC was 0.88 (95% CI: 0.72-1.00), indicating that this diagnostic model has a certain accuracy. Regarding prognosis, the enrolled patients are being tracked. The effect of differences in intestinal microbiota between MM patients in high-altitude and plain areas on disease prognosis will be in future research.

To explore the mechanism underlying the association between intestinal microbiota and MM, Calcinotto *et al* (24) used Vk*MYC mice to simulate human MM. They found that *Prevotella heparinolytica*, a gut commensal bacterium, can promote the differentiation of Th17 cells that produce IL-17 and that Th17 cells can migrate to the bone marrow to promote disease progression. This study suggests that commensal bacteria in the gut unleash a paracrine signaling network between innate and adaptive immunity that promotes the progression of MM. The complement system is the major participator of adaptive immunity and previous studies have shown differences in complement levels between patients with MM and healthy populations (25-27). Similarly, the clinical indicator correlation analysis of the present study suggested that complement components are related to the composition of the intestinal microbiota in patients with MM. Jian *et al* (28) found that the accumulation of urea nitrogen due to renal damage during MM progression may result in the enrichment of nitrogen-cycling bacteria such as *Klebsiella* and *Streptococcus*, suggesting strong metabolic interactions between intestinal bacteria and the host. Patients with MM achieve negative minimal residual disease (MRD) after early treatment and often have improved outcomes than those who remain as MRD^+. Pianko *et al* (29) observed a higher relative abundance of *Eubacterium hallii* and *Faecalibacterium prausnitzii* in 16 MRD^- patients compared with 18 MRD^+ patients, which suggests that there may be a relationship between intestinal microbiota composition and treatment outcome in MM. Functionally, these two bacteria produce short-chain fatty acids (mainly butyric acid) that play anti-inflammatory roles. El Jurdi *et al* (30) found that the composition and abundance of the gastrointestinal microbiome changed following HSCT in patients with MM. Enrichment of *Blautia* and *Ruminococcus* was associated with a higher incidence of diarrhea, nausea and vomiting following transplantation. It is noteworthy that these two bacteria are anaerobic and do not produce butyric acid. Hu *et al* (31) showed significant temporal differences in the diversity and abundance of *Bifidobacterium*, *Prevotella*, *Sutterella* and *Collinsella* between MM patients with complete or partial remission after chimeric antigen receptor T cell therapy. Metabolomic analysis showed that intermediates involved in multiple amino acid metabolic pathways, such as choline, L-cysteine, rosmarinic acid, L-phenylalanine and 2-phenylacetamide were significantly enriched in patients in complete remission. The present study predicted multiple metabolic pathways related to MM through KEGG functional analysis, including not only central carbon and choline metabolism in cancer, but also pathways such as dibasic acid metabolism, apoptosis, infection and glycolysis/gluconeogenesis. The present study further enriched the signaling and metabolic pathways of the intestinal microbiota that affect MM progression.

In recent years, the introduction of new drugs such as proteasome inhibitors has extended the survival time of most patients with MM, which remains incurable in most cases. However, research on the pathogenesis of MM is limited. No substantive breakthroughs in treatment methods have been achieved to date. The present study conducted a diversity analysis and differential bacterial screening of the intestinal microbiota of patients through 16s rRNA high-throughput sequencing, which may aid in the development of clinical microbial markers. Moreover, the present study predicted the signaling and metabolic pathways of the intestinal microbiota related to MM using functional analyses.

However, there are some shortcomings in the present study. Due to the small number of enrolled patients, it did not classify and compare MM patients of different subtypes, nor did it analyze the relationship between different stages and prognosis in MM. The present study only described the differences between MM patients and healthy individuals and these deficiencies will remedied in future work. Although the present study represented a step forward in understanding the pathogenesis of MM, conclusive evidence is lacking. Metagenomics and metabolomics research will be conducted in the future to elucidate the molecular mechanisms by which the intestinal microbiota affects the occurrence and development of MM and to identify therapeutic targets for MM. With an in-depth study of intestinal microbiota, the diagnosis and treatment of MM should lead to substantive breakthroughs.

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Availability of data and materials

The data generated in the present study may be found in the NCBI Sequence Read Archive (SRA) database under accession number (PRJNA1090483) or at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1090483.

Authors' contributions

All authors contributed to the conception and design of this study. Material preparations were performed by XuG and HJ. Sample collection was performed by LS and YW. Related experiments were performed by LD and XiG. Data collection and analysis were performed by YK. The manuscript was written by XL. Funding and supervision were conducted by HG. XL and HG confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Hospital of Qiqihar (approval no. 2021-KY-007-04). All patients signed written informed consent in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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