Bacteroides, butyric acid and t10,c12-CLA Changes in Colorectal Adenomatous Polyps

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Research

Keywords: Colorectal adenomatous polyps, microbiome, 16S rRNA, SCFA, bile acid, CLA

DOI: https://doi.org/10.21203/rs.3.rs-40596/v1

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Abstract

Background

Colorectal adenomatous polyps (CAPs) are considered precancerous lesions of colorectal cancer (CRC). There are few studies on patients with CAPs, and studies on microbial communities in the intestinal tract of CAP patients are inconsistent. The gut microbiota participates in the process of digestion and, in the process, produces metabolites, mainly short-chain fatty acids (SCFAs), secondary bile acids and conjugated linoleic acid (CLA). These studies aimed to investigate the gut microbiota and metabolites in the faeces of CAP patients in order to identify microbiota or metabolites that can be used as sensitive biological predictors and to provide a theoretical basis for the clinical treatment of CAPs.

Results

faeces Weissella and Lactobacillus were present in the HC group, and the abundance of Bacteroides and Citrobacter was higher in the CAP group than in the HC group. The expression of butyrate-producing bacterial genes was significantly higher in the faeces of CAP patients, but that of secondary bile acid-producing bacterial genes and CLA-producing bacterial genes showed no differences in either faeces or biopsy tissues, and that of butyrate-producing bacterial genes showed no differences in biopsy tissues. The contents of acetic acid and butyric acid were increased in the faeces of the CAP group, and the HC group had higher contents of t10,c12-CLA.

Conclusion

The results of gut microbiota in faeces showed no specific correlation between CAP patients and healthy individuals, but there was indicate that changes of specific genus might be detrimental to intestinal health. In addition, t10,c12-CLA played an important role in protecting the intestine.

Background

Colorectal cancer (CRC) is a common malignant tumour of the digestive tract, with the third highest incidence and second highest mortality among tumours worldwide [1, 2]. Colorectal adenomatous polyps (CAPs) are regarded as a critical precursor to CRC[3], and adenoma is an early neoplastic tissue that has not gained the properties of a cancer.

There are 100 trillion bacteria in the human intestine, and the collective genome of these bacteria is called the gut microbiome, which is 150 times the size of the human genome[4]. The existence of gut microbiota is essential for the growth and physical health of the human body. The gut microbiota participates in digestion and absorption of food in the intestines and actively participates in cell-mediated immune responses, which maintain the intestinal barrier function and maintain the stability of the intestinal
environment. The imbalance of this symbiotic relationship might have an adverse effect on the host, and gut microbiota imbalance has been observed in cases of inflammatory bowel disease (IBD)[5], obesity (Difference in the Gut Microbiome between Ovariectomy-Induced Obesity and Diet-Induced Obesity) [6], and cancer[7].

Moore et al.[8] applied culture methods to analyse the faeces of CRC patients and colorectal polyp patients and found that the abundance of *Bacteroides* and *Bifidobacteria* was positively correlated with the risk of colon polyps, while that of *Lactobacilli* and *Eubacteria* was related to the intestinal tract and had a protective effect. However, the types of bacteria that could be cultured in faeces were limited, and most of the bacteria could not be cultured in an in vitro environment. Therefore, the emergence of high-throughput sequencing technology and metagenomic analysis provided a better solution for analysing complex microbiome data.

SCFAs have the function of shaping the intestinal environment, affecting the physiology of the colon, and are the main energy source for host colon cells[9]. Studies have shown that butyrate plays a complex role in colorectal cancer, and this role might be related to the butyrate concentration in the colon environment. Bile acids are involved in cholesterol metabolism, lipid digestion, and host-microbial interactions in the human body and play an important role in the regulation of bile intestinal hepatic circulation[10]. The secretion of bile acids is closely associated with smoking, drinking, and a high-fat diet. Conjugated linoleic acid (CLA) is a health-promoting fatty acid with anti-cancer, anti-atherosclerosis, anti-diabetes, anti-obesity, anti-allergic and immune-enhancing properties. The anti-cancer properties of CAL in vivo and in vitro have been widely recognized[11].

To verify the profile of gut microbiota in CAP patients, we collected faeces from CAP patients attending the First Affiliated Hospital of Kunming Medical University. High-throughput sequencing technology was used to analyse the gut microbiota in the intestinal tract, which made the understanding of the microbial community more complete. Analysis of the metabolites in the faeces of CAP patients was performed to identify the metabolites that changed and to explore the changes in the gut microbiota and its metabolites during the progression of CAP to CRC. This study will provide a comprehensive information about gut microbiota and metabolites changing in CAP patients, which help to characterize the role of gut microbiota and metabolites in adenoma happening and progressing, and the differences of gut microbiota and metabolites might consider as biomarker of CAP in future.

**Results**

**Changes in gut microbiota in the faeces of CAP patients.** Four unqualified samples were deleted, a total of 9433 OTUs (operational taxonomic units) were generated, with an average of 168 OTUs per sample, and the library coverage of all samples was over 99.9%. It can be considered that the sequencing depth covered all species in the samples.

The α diversity analysis indicated that the Chao, Ace, Shannon and Simpson indexes were not significantly different between the two groups (Supplementary Fig. 1). To observe the difference in
composition of the two samples by PCoA analysis (Fig. 1), in which the PC1 coordinates represented the main coordinate component that caused the largest difference in samples; PC1 explained 21.4% of the difference, followed by PC2, explaining 12.01%. In the PCoA diagram, it was observed that the samples in the CAP group and HC group were not completely separated, and some samples were aggregated.

Analysis of bacteria at the phylum level. A total of 12 bacterial phyla were found in the CAP and HC samples. The phyla with the highest proportions in the two groups were *Firmicutes* (CAPs, 52.07%; HCs, 55.32%), *Bacteroidetes* (CAPs, 26.93%; HCs, 23.44%) and *Proteobacteria* (CAPs, 20.52%; HCs, 19.32%). Overall, the difference in *Cyanobacteria* was statistically significant (*P < 0.01*) (Wilcoxon test) (Fig. 2).

Analysis of bacteria at the genus level. A total of 121 bacterial genera were found in the CAP group and HC group, of which the highest proportions were *Bacteroides* (CAPs, 22.51%; HCs, 15.28%), *Escherichia* (CAPs, 14.50%; HCs, 15.83%) and *Faecalibacterium* (CAPs, 12.45%; HCs, 13.60%). Among all genera, the abundance of *Citrobacter* (*P < 0.05*) and *Bacteroides* (*P < 0.05*) increased in the CAP group, and the abundance of *Erwinia* (*P < 0.01*), *Weissella* (*P < 0.01*), *Lactobacillus* (*P < 0.05*) and *Peptoniphilus* (*P < 0.05*) decreased in the CAP group (Fig. 3).

Weighted UniFrac analysis of species phylogenetic evolution. Differences in the diversity between the two groups of samples, including the weighted UniFrac analysis of OTU abundance considerations and the observation of OTU abundance in the heatmap, were compared with β diversity analysis (Fig. 4). The distance between samples was changed, but the differences were not significant.

**Changes in metabolites in CAP patients.** The results of SCFA, bile acid and CLA showed acetic acid and butyric acid increased, meanwhile *t10,c12-CLA* decreased in faeces of CAP patients (*P < 0.05*) (Table 2). Operating characteristic curve (ROC) describing the prediction accuracy of these three different metabolites and *Bacteroides*. The area under the curve of *Bacteroides*, acetic acid, butyric acid and *t10,c12-CLA* were 0.648, 0.704, 0.781 and 0.203 respectively(Fig. 5).

| Table 1 | Demographic information |
|----------|------------------------|
|          | CAP (n = 28) | HC (n = 28) | P-value |
| Age      | 53.23 ± 10.14 | 50.33 ± 10.87 | 0.287   |
| Gender (male/female) | 20/10       | 13/17      | 0.069   |
| BMI (kg/m²) | 24.77 ± 2.00 | 24.48 ± 1.83 | 0.451   |
Table 2
Metabolites analysis and comparison of CAP patients and healthy volunteers

|                  | CAP (n = 28)       | HC (n = 28)       | P-value |
|------------------|--------------------|--------------------|---------|
| acetic acid      | 596.24 ± 176.54    | 468.27 ± 171.63    | 0.003   |
| propionic acid   | 193.15 ± 81.10     | 166.60 ± 69.92     | 0.186   |
| isobutyric acid  | 10.49 ± 6.99       | 8.73 ± 10.85       | 0.090   |
| butyric acid     | 300.09 ± 186.32    | 143.87 ± 95.79     | 0.000   |
| isovaleric acid  | 8.72 ± 4.97        | 12.08 ± 14.92      | 0.941   |
| valeric acid     | 23.55 ± 20.47      | 25.11 ± 18.82      | 0.7902  |
| CA               | 35.91 ± 67.93      | 39.38 ± 59.67      | 0.241   |
| CDCA             | 36.51 ± 34.10      | 35.56 ± 49.02      | 0.22    |
| DCA              | 52.61 ± 58.92      | 29.27 ± 32.22      | 0.22    |
| LCA              | 12.52 ± 14.64      | 18.76 ± 24.15      | 0.45    |
| c9,t11-CLA       | 108.96 ± 125.42    | 173.33 ± 200.50    | 0.234   |
| t10,c12-CLA      | 11.28 ± 14.96      | 17.90 ± 13.06      | 0.013   |

**Analysis of qPCR with metabolites produced by bacteria.** The dissolution curve and amplification curve of the internal reference gene and the target gene were observed to be complete, smooth and without peaks, indicating that the specificity of the amplification products was good (Supplementary Table 1).

In stool samples, the gene expression of butyrate-producing bacteria in the CAP group was lower than that in the HC group, but the gene expression of bile acid-producing bacteria and conjugated linoleic acid-producing bacteria in the CAP group was not significantly different from that in the HC group (Fig. 6A-C).

**Discussion**

There are complex microbial inhabited in human intestine, and the group maintained the stability of intestinal environment and protect the health of human body. These microbial participate in defense and immunity against pathogens, development of intestinal microvilli, and ferment non-digestible dietary fibers, anaerobic metabolism of peptides and proteins, providing energy to the host. A number of studies have shown that the gut microbiome is associated with the occurrence of CAPs. However, most intestinal bacteria cannot be cultured in vitro, the application of high-throughput sequencing technology helped us fully understand how the gut microbiome changed during the development process from healthy to CAP and CRC.
In the 16S rRNA sequence analysis of faeces, the alpha diversity index was not statistically significant between the two groups. Goedert et al. reported similar results of faecal microbiota in CAP patients[12], but a reduction in the abundance of faecal microbiota was observed in CRC patients[13]. PCoA analysis of weighted UniFrac revealed no obvious aggregation in the CAP group and HC group. The changes in genera in both groups were analysed, and *Weissella* and *Lactobacillus* were present in the HC group, although the relative content was not high, but *Weissella* and *Lactobacillus* are probiotics[14]. The difference in the two groups indicated that the two genera might have a protective effect on the intestine. In addition, the abundances of *Bacteroides* and *Citrobacter* in the CAP group were higher than those in the HC group, which indicated that these two species might play an important role in the pathological process of CAP. Studies have found that *Citrobacter* can take over the “cell-cell” communication system to trigger colitis in mice[15], and the elevation of *Bacteroides* in the faeces of CAP patients has been confirmed[5]. Compared with the bacteria in faeces, the bacteria attached to colonic mucosa were more likely to affect the gene expression of colonic mucosa cells. Based on high-throughput sequencing of biopsy tissue[16], the alpha diversity of polyps was higher than that of healthy tissue, which indicated that polyps have higher within-habitat diversity than does healthy tissue. This phenomenon of increased diversity also appeared in studies on CRC[17], which might suggest that increased diversity of the gut microbiome is not a sign of healthy intestines but rather the excessive growth of various harmful bacteria or archaea in adenoma and cancer development[3]. Studies on the faeces and tumour tissues of CRC patients have shown different results for *Bacteroides*[18–20]. Yu et al.[21] found that the abundances of *Proteobacteria* and *Fusobacteria* were high in tumour tissues of CRC patients, but in this study, there were no significant differences in the two groups of bacteria. Many studies have found *Fusobacteria* enriched in the faeces and tumour tissues of patients with CRC[22], but there were no significant differences in the abundance of *Fusobacteria* in faeces or adenoma tissues between CAP patients and healthy controls. The relative content of *Fusobacteria* in faeces and polyps was low, and it was speculated that the enrichment of *Proteobacteria* may be related to the degree of tissue abnormality.

In this study, the acetic acid and butyric acid contents in the faeces were higher in the CAP group than in the HC group. Butyrate, as a major source of energy for intestinal epithelial cells, could reduce colonic inflammation, induce apoptosis, inhibit tumour cells and prevent CRC development. The antiproliferative and anticancer properties of butyrate have been demonstrated and are probably attributable to the effect of high concentrations of butyrate as a histone deacetylase inhibitor (HDACi)[23]. However, Bultman et al.[24] believed that butyrate is a causative factor of CRC, and a study on APC\(^{Min/+}\)MSH2\(^{-/-}\) mice fed butyrate showed that the amount of butyrate administered is positively correlated with the formation of polyps in mice, which might be due to the stimulation of hyperproliferation of the gut microbiota and cell transformation of mouse intestinal epithelial cells through metabolites. The formation of polyps at low concentrations stimulates the proliferation of colonic epithelial cells[25]. These opposing effects of butyrate have been called the "butyrate paradox". Although the propionic acid content was not significantly different between the two groups, the propionic acid content in the CAP group was increased, and *Bacteroides*, which is a major contributor to propionate synthesis, was significantly abundant in the CAP group. Due to the different positions and conformations of the conjugated double bonds, there were
multiple isomers of conjugated linoleic acid and the main ones being $c_9,t_{11}$-CLA and $t_{10},c_{12}$-CLA [26], these two isomers played different roles in anticancer and antcardiovascular disease activity. Here, $t_{10},c_{12}$-CLA was found to be increased in the faeces of the HC group, but the difference in $c_9,t_{11}$-CLA content between groups was not statistically significant. CLA has functions such as reducing body fat, restricting tumour development, preventing cardiovascular disease and improving immunity [27]. As a fatty acid that protects the intestine, its antitumour properties in vitro and in vivo have been widely recognized [11]. Among the isomers, $t_{10},c_{12}$-CLA has functions of reducing body fat, lowering triglyceride content and inhibiting adipocyte differentiation, and $t_{10},c_{12}$-CLA was found to be more effective in inhibiting tumours than were other isomers. The effects of inhibiting tumour cell growth were positively correlated with its concentration, and $c_9,t_{11}$-CLA played an important role in immune regulation [28–30]. In addition, $t_{10},c_{12}$-CLA showed a significant decrease in the CAP group, and it was considered that the decrease in $c_9,t_{11}$-CLA content in faeces might increase the risk of intestinal adenomatous polyps. A high-fat diet strongly stimulates bile acid production, and bile acids are converted to secondary bile acids (DCA and LCA) after structural modification of bacteria with $7\alpha$-dehydroxy activation in the gut. DCA is the most typical secondary bile acid [13]. Secondary bile acids promote the proliferation of intestinal epithelial cells, induce apoptosis and mutation, and promote cancer progression [31]. There were no significant differences in the levels any bile acids in our study, but the content of DCA was higher in the CAP group. Lu et al. [32] found that CDCA and DCA showed a significant increase in faeces only in CRC patients, but no significant differences were observed in CAP patients. The gut microbiota converts primary bile acids into secondary bile acids, suggesting that the gut microbiota can affect the composition of secondary bile acids, while changing the profile of secondary bile acids could reshape the composition of intestinal bacteria [33]. Although there was no significant difference in DCA or CDCA between groups, the abundance of Bacteroides, which has bile acid-resistant characteristics, was positively correlated with fat and protein intake [24, 34, 35], and the abundance of Bacteroides showed a significant increase in the CAP group.

There are researches indicate the relationship between gut microbiota and metabolites in intestinal track. The ability of gut microbiota to produce metabolites such as butyrate, secondary bile acid and CLA can vary the regulation of gut environment, as has been shown in response to diet. This study aimed to investigate if there were gene expression differences between CAP patients and healthy volunteers. In analysis of DNA from faeces, the expression of butyrate-producing bacterial genes in the CAP group was significantly lower than that in the HC group, but there was no significant difference of DNA between the two groups. The result indicated that the abundance of butyrate-producing bacteria in the faeces of CAP patients was decreased, while the content of butyric acid in the CAP group was higher than that in the HC group. Therefore, the concentration of SCFAs in faeces does not fully reflect the concentration of SCFAs produced by gut microbiota fermentation; thus, the intestinal health effects need to be carefully considered [36]. SCFAs can effectively reduce the pH of the intestine, promote the glycolysis of food in the intestine and reduce the absorption of carcinogens, which can reduce the risk of CRC [37]. The differences in secondary bile acid-producing bacteria and CLA-producing bacteria were not statistically significant in this study. Mullish et al. [38] have found that baiCD operon was not in all bacteria with $7\alpha$-
dehydroxylating ability, which has been considered to an important process for the formation of secondary bile acids in faeces[11]. Certain species of *Bifidobacteria* in the gut, as natural colonizers, were capable of converting linoleic acid to \( c_9t_11\text{-CLA} \), \( t_{10}c_{12}\text{-CLA} \) and small amounts of \( t_9t_{11}\text{-CLA} \) [39]. There was no statistically significant difference in \( c_9t_{11}\text{-CLA} \) content in faeces between the two groups, and it was considered that the abundance of *Bifidobacteria* that produce \( c_9t_{11}\text{-CLA} \) was not significantly different in the two groups.

Metabolomics provide a qualitative and quantitative method of metabolites in analysis which can complete analysis with microbiology. Metabolites (small molecules < 1500 Da) are intermediates or end products of cellular metabolism, which can be produced directly by the host organism, or can be derived from various other external sources such as diet, microbes, or xenobiotic sources[40]. Biological systems are complex and analytical limitations, it is not possible to identify all the metabolites present in a specimen. Researches about metabolites and diseases indicate that diabetes[41], cardiovascular disease and heart failure[42, 43], autism[44] and anxiety[45]. With technology and researches progressing, more and more evidence would show that specific species of gut microbiota and metabolites play an important role in disease, which would be biomarker for disease diagnose in future.

In this study, the detection of gut microbiota and metabolites indicated that there still existed something insufficient. First of all, the structure of sample disease stage were unbalanced, the proportion of high-grade intraepithelial neoplasia were lower than others samples. It is necessary to expand sample size, elevate the proportion of high-grade intraepithelial neoplasia. Second, the direct interaction between gut microbiota and metabolites, that the proportion of specific gut microbiota and its related metabolites content also need further research. Third, the effect of food and antibiotics intake on intestinal microenvironment, and its influences on disease occurrence and progression is a promising study. Nowadays, with technology developing, the detect limitation would be solved and have multiple vision on correlation between gut microbiota and metabolites in future.

**Conclusion**

In present study indicated gut microbiota analysis in faeces showed that the abundance of *Weissella* and *Lactobacillus* were decreased, and *Bacteroides* and *Citrobacter* increased in the CAP group. Metabolites detection showed changes on butyrate and \( t_{10}, c_{12}\text{-CLA} \) indicate that more experiment need to investigate the function would be protect or damage in intestinal environment. Analysis on gut microbiota carried operon of producing metabolites demonstrate there might be more functional operon exist in gut microbiota or intracellular. With these researches on gut microbiota and metabolites, the changing abundance of gut microbiota and metabolites content would be biomarkers in disease diagnosis, which provide a convenient way to protect intestinal health.

**Methods**
Sampling. Thirty patients with colorectal adenomatous polyps were selected from the First Affiliated Hospital of Kunming Medical University from November 2017 to April 2018. Exclusion criteria included obesity, special eating habits, colorectal cancer, history of colorectal surgery, colitis (ulcerative, Crohn's), metabolic disease (diabetes, hyperlipidaemia), and infectious disease. Thirty healthy volunteers were selected from the First Affiliated Hospital of Kunming Medical University as controls. No subjects were taking antibiotics, immunosuppressive agents, corticosteroids or probiotics within 3 months prior to sample collection. There were no significant differences in age, gender, or BMI between the two groups (P> 0.05) (Table 1). Stool samples from volunteers were preserved after admission, and samples (CAPs) were collected in accordance with the relevant guidelines and regulations.

DNA extraction and 16S rRNA gene amplification. Genomic DNA was extracted from stool samples and biopsy samples with the QIAamp DNA Stool Mini Kit. The primers for amplification of the V3-V4 region of the bacterial 16S rRNA gene, after amplification high throughput sequencing was performed with the Illumina MiSeq platform.

Bioinformatics Analysis. The high-quality paired-end reads were combined to tags based on overlaps, and the consensus sequence was generated by FLASH (Fast Length Adjustment of Short reads, v1.2.11). The tags were clustered into OTUs (operational taxonomic units) by scripts in USEARCH software (v7.0.1090), OTUs were clustered with a 97% similarity cutoff using UPARSE, and OTU representative sequences were taxonomically classified using the Ribosomal Database Project (RDP) Classifier v.2.2 trained on the Greengene_2013_5_99 database with a 0.6 confidence value as the cutoff. OTUs were filtered by removing unassigned OTUs and removing OTUs not assigned to target species. OTUs were used for alpha diversity estimation. Comparison of the beta diversity, which is the difference in species diversity between two groups, was performed based on the OTU abundance by QIIME (V1.80). Specimen annotation analysis is a method that compares OTUs to a database of classified OTUs at the phylum, class, order, family and species levels, and the analysis is then presented by histograms. UniFrac analysis used phylogenetic information to compare species community differences between samples. Metabolite determination Ion Chromatograph Analysis. The faecal samples stored in the refrigerator at -80°C were removed, weighed to 300 mg, dissolved in 1 mL of dH2O, vortexed and mixed for 30 s. Then, the supernatant was incubated with a 0.22 μm microporous nylon membrane (water system). The liquid was filtered, placed in an EP tube and placed in a refrigerator at -20°C for use. Standard curves were generated using standard solutions. A volume of 25 μL was injected into a chromatographic column (DIONEX IonPac AG11-HC 4×50 mm & IonPac AS11-HC 4×250 mm, USA) and eluted with KOH at a flow rate of 1.2 mL/min; ions were detected by a conductivity detector in an ion chromatograph (Thermo Dionex ICS-3000, USA), and the column temperature was 30°C. UPLC-MS/MS Analysis. The faecal samples stored in the refrigerator at -80°C were removed, weighed to 200 mg, dissolved in 1 mL of methanol, vortexed and mixed for 30 s. The supernatant was then incubated with a 0.22 μm microporous nylon membrane (organic system). The liquid was filtered, placed in an EP tube and placed in a refrigerator at -20°C for use. Standard curves were generated using standard solutions. UPLC conditions. The mobile phase consisted of 0.05% ammonia (5 mM aqueous solution) in water as solution A and acetonitrile as solution B. The flow rate of the mobile phase through the column was 0.4 mL/min (Waters BEH C18 1.7 μm, 50×2.1 mm, USA) at a temperature of 40°C. The injection volume was 1 μL. Mass spectrometry conditions included electrospray ionization, negative ion mode, multiple reaction detection, air as the
desolvation gas, nitrogen as the cone gas, and argon as the collision gas. Real-time PCR analysis To explore bacteria that produce specific metabolites, real-time PCR was used (TIANLONG Gentier 96, Xi’an, China). Real-time PCR experiments were performed with GoTaq Green Master Mix (Promega, USA) in a total volume of 20 μL. The amplification cycle used was 1 cycle of 95°C for 2 min; 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 min each with data acquisition at 72°C; 1 cycle of 72°C for 10 min; and cooling to 4°C. To obtain the Ct value of samples, 2-△△Ct was calculated for statistical analysis by using SPSS 22.0 and GraphPad Prism 7.0.

Declarations

Ethical approval This research was approved by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University ((2017) L-15). All volunteers signed informed consent.

Availability of data and materials The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests The authors declare that they have no competing interests.

Funding This work was supported by funding from the National Natural Science Foundation of China (NO. 81960382); Natural Science Foundation of Yunnan Province (NO. 2018FA043); Joint special fund for applied basic research in Yunnan Province (NO. 2017FE467 (-173)) and (NO.2019FE001 (-060)); Training plan for medical discipline leaders of Yunnan health and family planning Commission (NO. D-2017023).

Authors’ Contributions Ciyan Chen, Min Niu, and Shumin Liu performed the data analysis and interpretation; Yong Duan and Yan Du participated in the discussion and interpretations of the results; Ciyan Chen, Huanqin Li, Qiu Yue He, and Jian Mao drafted the manuscript; and Yan Du designed the study and revised the manuscript. All authors approved the submission.

Acknowledgements We thank Dr. Yinglei Miao (the First Affiliated Hospital of Kunming Medical University) for technical advice, Dr. Yang Sun (the First Affiliated Hospital of Kunming Medical University) for assistance with sample collection and high-throughput sequencing analysis, and Dr. Junxi Pan (the First Affiliated Hospital of Kunming Medical University) for experiment guidance. The authors express their gratitude to the anonymous reviewers for their valuable comments and suggestions.

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Figures
**Figure 1**

PCoA analysis: PC1 coordinates represent the main coordinate component that caused the largest difference in the sample, and PC2 represents the second coordinate component.
Figure 2
Taxonomy bar plot of abundance at the phylum level

Figure 3
Taxonomy bar plot of abundance at the genus level; different colours represent different bacterial phyla.
Figure 4

Weighted UniFrac analysis of the phylogenetic relationships of species.
Figure 5

ROC curve describing the prediction accuracy of four particular genus and metabolites, with AUC values.
Figure 6

Gene expression comparison of fecal from two groups. NS: None significantly differences; **: P<0.05.

Supplementary Files

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