TCM syndrome differentiation in colorectal cancer patients assisted by differences in gut microbiota: An exploratory study

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ABSTRACT

Objective: To explore the difference in gut microbiota between different traditional Chinese Medicine (TCM) syndromes in patients with colorectal cancer (CRC) and its internal relationship.
Methods: From June 2020 to August 2021, 109 colorectal cancer patients with a clear pathological diagnosis who had not yet undergone surgery or chemotherapy were classified according to the TCM syndrome classification, and the feces samples of 109 patients with preoperative colorectal cancer were collected. 16s rRNA gene sequencing was used to determine gut microbiota diversity and abundance in CRC patients with different TCM syndrome, and LEfSe analysis was made to screen different TCM syndrome for differential representative microbiota.
Results: 109 patients were divided into 5 syndromes by TCM syndrome classification, which were Liver and Kidney Yin Deficiency Syndrome (LKYDS, n = 19), Spleen Deficient Qi Stagnation Syndrome (SDQSS, n = 30), Stasis and Poison Obstruction Syndrome (SPOS, n = 17), Damp-Heat Syndrome (DHS, n = 30), Qi and Blood Deficiency Syndrome (QBDS, n = 13). Alpha diversity index showed significant differences among the five groups of TCM syndromes, with Shannon index being highest in the SDQSS group and lowest in the LKYDS (p = 0.003). ACE index being highest in the SDQSS group and lowest in the SPOS (p = 0.010). PD whole tree index being highest in the SDQSS group and lowest in the SPOS (p = 0.017). Similarly, beta diversity showed significant differences among the five groups of TCM syndromes, with principal coordinate analysis (PCo1 = 31.86 %, PCo2 = 5.62 %) showing separation and coincidence between the groups, and Adonis group differences showing coincidence between the QBDS-LKYDS (p = 0.702), QBDS-DHS (p = 0.133), and SDQSS-DHS (p = 0.260) groups. LEfSe analysis revealed that the representative microbiota of DHS patients was *Dialister* sp *Marseille P5638* (LDA = 3.05, p < 0.001), the representative microbiota of SPOS patients was *Oscillospirales* (LDA = 4.78, p = 0.029), the representative microbiota of SDQSS patients was *Selenomonadaceae* (LDA = 3.94, p = 0.003), the representative microbiota of LKYDS patients was *Dialister* (LDA = 4.19, p = 0.001), and the representative microbiota of QBDS patients was *Akkermansia muciniphila* (LDA = 4.23, p = 0.006).
Conclusions: There are significant differences in gut microbiota between different TCM syndromes in CRC patients. The five microbiota, *Dialister* sp *Marseille P5638*, *Oscillospirales*,

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Selenomonadaceae, *Dialister*, and *Akkermansia muciniphila*, may be differential markers of TCM syndrome in CRC and are expected to be one of the bases for accurate TCM syndrome differentiation of CRC.

1. Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies worldwide [1]. Similarly, the incidence of CRC in China has also shown an increasing trend year by year, and the affected population gradually tends to be younger. In recent years, although China has achieved good clinical efficacy in the treatment of CRC. Surgery, radiotherapy, chemotherapy and other treatment measures prolong the overall survival and quality of life of patients with CRC to a certain extent, but there is still a certain gap in clinical efficacy compared with developed countries such as Europe and the United States. This has also led researchers to explore newer, more comprehensive treatments, such as integrative medicine [2,3].

In China, TCM has been used to treat diseases for thousands of years, and TCM is considered to be a major treasure trove in China. A number of studies have confirmed the role of TCM in the treatment of neoplastic diseases and have obtained certain clinical benefits in clinical treatment [4,5]. "TCM syndrome type" is an objective reflection of the patient's body condition at the current disease stage. In the process of clinical diagnosis and treatment, TCM physicians use the method of "observing, listening, inquiring and pulse feeling" to carry out TCM syndrome differentiation for CRC patients, and carry out targeted "Chinese herbal medicine" treatment according to the patient's "TCM syndrome type" to achieve the purpose of treating CRC. Therefore, precise discrimination of TCM syndromes is a prerequisite for clinical treatment.

The gut microbiota is likened to a special "organ" in the human body, and its resident microbiota has more than 1000 species, including probiotics, pathogenic bacteria, and opportunistic pathogens, which are involved in the digestion, metabolism, energy conversion, and immune processes of the human body [6,7]. The development of CRC is closely linked to structural changes in the gut microbiota, and the mechanisms of impact are mainly related to chronic inflammation and immune function [8,9]. With the progress of TCM related research, the objective material basis of different TCM syndromes has gradually increased. *Streptococcus* and *Lachnoclostridium* in the gut microbiota have been found to be able to distinguish Pi-Xu-Shi-Yun syndrome and Da-Chang-Shi-Re syndrome in Ulcerative colitis as differential microbiota [10], and it has been confirmed that there are significant differences in the gut microbiota between CRC patients and healthy individuals [11]. However, differences in gut microbiota between different TCM syndromes in CRC patients have rarely been reported. The species and abundance of gut microbiota in the human body are constantly changing and maintain a dynamic balance with each other. This feature is very similar to the understanding of diseases in TCM, and TCM believes that the "vital qi" and "evil qi" in the population maintain each other in a dynamic balance state, and once this balance is broken, it may cause the occurrence of diseases (i.e., "imbalance between vital qi and evil qi"). Previous reports on TCM syndromes of CRC have shown that cancer patients' corresponding TCM syndrome deficiency syndrome significantly increased and excess syndrome significantly decreased after receiving surgery [12] and chemotherapy [13]. Therefore, in order to accurately understand the differences between gut microbiota in CRC patients with different TCM syndromes, in this study we excluded the effects of surgery and chemotherapy on TCM syndromes, analyzed different TCM syndromes before surgery and fecal gut microbiota in 109 CRC patients during the same period, and sought the link between TCM syndromes and gut microbiota in patients in order to use differential gut microbiota to assist TCM physicians in more accurate TCM clinical syndrome differentiation.

2. Data and methods

2.1. Clinical data

From June 2020 to August 2021, 109 feces samples were collected from patients with preoperative CRC who visited the Department of Colorectal and Anal Surgery of the 940th Hospital of Joint Logistics Support Force of Chinese People's Liberation Army. Among them, there were 38 cases of colon cancer and 71 cases of rectal cancer. There were 67 males and 42 females, aged 36–88 years, with an average age of 65 years. This research plan has been reviewed and approved by the Clinical Research Ethics Committee of the hospital (No.2020KYLL075).

2.2. Criteria inclusion and exclusion

2.2.1. Criteria for inclusion

(1) Patients with primary colorectal cancer confirmed by pathological diagnosis.
(2) Patients who live in Gansu Province for a long time.
(3) Patients who have not undergone surgery, neoadjuvant chemoradiotherapy, targeted therapy, etc.
(4) Patients who have been informed and have signed informed consent form.

2.2.2. Criteria for exclusion

(1) Patients whose dialectical results could not be agreed by three TCM physicians.
(2) Patients treated with antibiotics, immunosuppressive agents, Chinese herbal medicines and other drugs in the past three months.
(3) Patients who have undergone gastrointestinal surgery within five years.
(4) Patients with severe complications such as intestinal perforation, bleeding, obstruction at presentation.
(5) Pregnant or lactating women.

2.3. TCM syndrome differentiation

TCM syndrome differentiation was performed according to previous methods of our group [14]. Briefly, according to the six TCM syndromes specified in the Guidelines for TCM Diagnosis and Treatment of Tumors issued by China [15]. Three TCM physicians divided the enrolled patients into six TCM syndromes: Liver and Kidney Yin Deficiency Syndrome (LKYDS), Spleen Deficiency and Qi Stagnation Syndrome (SDQSS), Stasis and Poison Obstruction Syndrome (SPOS), Damp-Heat Syndrome (DHS), Qi and Blood Deficiency Syndrome (QBDS), and Spleen-Kidney Yang Deficiency Syndrome (SKYDS) using the four diagnostic information of TCM.

2.4. Queue design

The queue design of this study was shown in Fig. 1. Because only 4 patients were included in the SKYDS group, which did not meet the minimum biological repeat requirement for 16S rRNA gene sequencing, the SKYDS group was excluded from this study.

2.5. Sample collection, storage and transportation

Five grams of naturally excreted feces samples from enrolled patients were taken by the researcher into cryogenic vials on the morning of the next day of admission, transferred to a $-80\ ^{\circ}C$ freezer within 2 h for cryopreservation until examination, and finally transported under dry ice conditions to the testing company for further testing and analysis (Suzhou PANOMIX Biomedical Tech Co., Ltd. China).
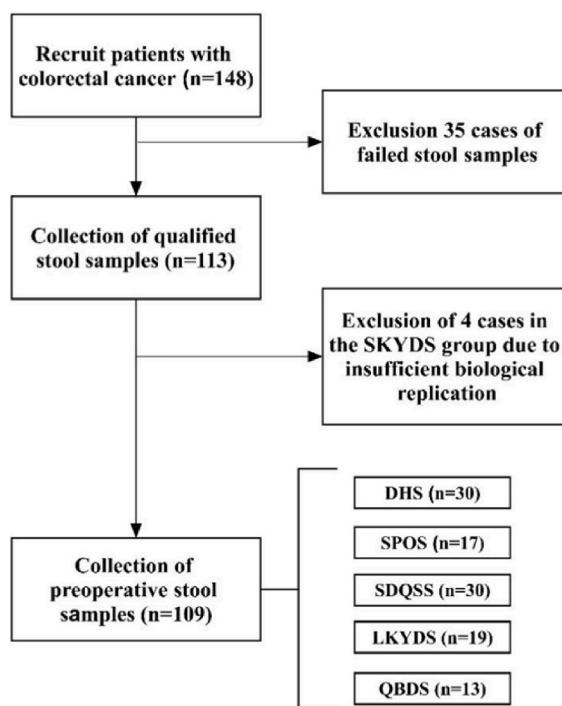

Fig. 1. Queue design.
Abbreviation: DHS: Damp-Heat Syndrome. SPOS:Stasis and Poison Obstruction Syndrome. SDQSS: Spleen Deficient Qi Stagnation Syndrome. LKYDS: Liver and Kidney Yin Deficiency Syndrome. QBDS: Qi and Blood Deficiency Syndrome. SKYDS: Spleen-Kidney Yang Deficiency Syndrome.

2.6. Sample sequencing

2.6.1. Extraction of genome DNA

Total genome DNA from samples was extracted using CTAB method. DNA concentration and purity were monitored on 1 % agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water.

2.6.2. PCR amplification

16S rRNA genes of distinct regions 16S V3–V4 were amplified used specific primer 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) with the barcode. All PCR reactions were carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs); 0.2 μM of forward and reverse primers, and about 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s. Finally 72 °C for 5 min.

2.6.3. PCR products quantification and Purification

Mix same volume of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2 % agarose gel for detection. PCR products was mixed in equidensity ratios. Then, mixture PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany).

2.6.4. Library preparation and sequencing

Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina NovaSeq 6000 platform by Panomix (Suzhou, Jiangsu, China).

2.7. Data processing and bioinformatics analysis

2.7.1. Data processing for sequencing

By using the software of FLASH (V1.2.7, Fig. http://ccb.jhu.edu/software/FLASH/), and QIIME software (V1.9.1, Fig. http://qiime.org/scripts/split_libraries_fastq.html), the sequencing data were spliced and filtered to obtain Clean Tags. The tags were compared with the reference database (Silva database, https://www.arb-silva.de/) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences, and then the chimera sequences were removed. Finally, the Effective Tags were obtained.

2.7.2. OTU clustering and taxonomic analysis

Sequences analysis were performed by Uparse software (V7.0.1001 , http://drive5.com/uparse/). Sequences with ≥97 % similarity were assigned to the same OTUs. For each representative sequence, the Silva Database (http://www.arb-silva.de/) was used based on Mothur algorithm to annotate taxonomic information. In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples (groups), multiple sequence alignments were conducted using the MUSCLE software (V3.8.31, http://www.drive5.com/muscle/). OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences.

2.7.3. Alpha, beta diversity and enterotypes analysis

Using QIIME software (Version 1.9.1, http://qiime.org/scripts/split_libraries_fastq.html), Alpha diversity indices, including Shannon index, Ace index, and PDwhole tree index, were calculated for all sample data. R software (Version 2.15.3) was used to draw dilution curves (Rarefaction Curve) and rank clustering curves (Rank abundance), and Alpha diversity indices were analyzed for differences between groups. Beta Diversity was used to analyze the gut microbiota community structure of different samples, Unifrac distance (Unweighted Unifrac) was calculated from the phylogenetic relationship between OTUs, and multivariate principal coordinate analysis and Adonis group difference analysis were made to investigate the differences in microbiota diversity between the groups. Samples were clustered using Jensen-Shannon distance and partitioning around medoid (PAM) clustering.Optimal number of clusters was estimated using CalinskiHarabasz (CH) index. Choose the number of clusters that maximizes the CH index as the optimal number of Enterotypes. Each sample is then assigned to one of the Enterotypes based on the cluster it belongs to in the PAM clustering results.

2.7.4. Functional Predictive analysis

The KEGG database prokaryotic whole genome 16S rRNA gene sequences were extracted and aligned to the SILVA SSU Ref NR database (BLAST bit score >1500) using the BLASTN algorithm to establish a correlation matrix. Then the genome-wide functional information of prokaryotes annotated by two methods, UProC and PAUDA, was mapped to the SILVA database for SILVA database functional annotation.

2.7.5. Linear discriminant analysis effect size (LEfSe) analysis

In this study, the screening value of linear regression analysis value (LDA Score) was set as 3 and $P < 0.05$, and linear discriminant

analysis was performed using LEfSe software. In the first place, the Kruskal-Wallis rank sum test was used to detect species with significant abundance differences between groups in the gut microbiota of CRC patients with five different TCM syndromes, and then the Wilcoxon rank sum test was used to analyze whether all subspecies in the differential species converged to the same taxonomic level. Finally, linear discriminant analysis was carried out to reduce and maintain the resulting data to assess the influence of differential microbiota.

2.8. Statistical analysis

All the data were analyzed by SPSS 25.0. The χ^2 test was used for statistical analysis of enumeration data. Statistical analysis of measurement data was performed using the *t*-test. Kruskal-Wallis H rank sum test was used to compare multiple groups, and the Wilcox rank sum test was used to test two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. General information

A total of 109 preoperative CRC cases were collected in this study, 67 males and 42 females, aged 36–88 years, with an average age of 65 years (64.64 ± 11.58). Among them, there were 30 patients in DHS group, 17 patients in SPOS group, 30 patients in SDQSS group, 19 patients in LKYDS group and 13 patients in QBDS group. Twenty-four patients (80 %) were male and 6 (20 %) were female in DHS group, 13 (76.5 %) were male and 4 (23.5 %) were female in SPOS group, 17 (56.7 %) were male and 13 (43.3 %) were female in SDQSS group, 7 (36.8 %) were male and 12 (63.2 %) were female in LKYDS group, and 6 (46.2 %) were male and 7 (53.8 %) were female in QBDS group. Informed consent was obtained from all volunteers prior to participation in the trial. There were no significant differences (p > 0.05) in clinical factors such as age, tumor location, TNM stage, and presence of metastasis in all the subjects except sex (p = 0.015) (Table 1).

3.2. Original sequence data statistics and quality control

In this study, after statistical processing of the raw data of each group of samples, 85,573 tags (PE reads) were detected in each

Table 1
Comparison of Clinical features of CRC patients with different TCM syndromes.

Clinical features	DHS (n = 30)	SPOS (n = 17)	SDQSS (n = 30)	LKYDS (n = 19)	QBDS (n = 13)	p
Gender, n (%)						0.015[a]
Male	24 (80.00)	13 (76.47)	17 (56.67)	7 (36.84)	6 (46.15)	
Female	6 (20.00)	4 (23.53)	13 (43.33)	12 (63.16)	7 (53.85)	
Age (Years)	61.20 ± 12.88	65.76 ± 7.94	66.60 ± 1.52	65.95 ± 12.88	67.46 ± 12.53	0.526
BMI (kg/m^2)	23.22 ± 3.51	23.13 ± 3.39	23.50 ± 3.39	23.56 ± 2.73	22.46 ± 4.37	0.067
Drinking, n (%)						0.529
Yes	7 (23.33)	1 (5.88)	6 (20.00)	2 (10.53)	2 (15.38)	
No	23 (77.67)	16 (94.12)	24 (80.00)	17 (89.47)	11 (84.61)	
Smoking, n (%)						0.239
Yes	10(33.33)	6(35.29)	6(20.00)	3(15.79)	1(7.69)	
No	20(66.67)	11(65.71)	24(80.00)	16(84.21)	12(92.31)	
Tumor site, n (%)						0.139
Right colon	6 (20.00)	2 (11.76)	2 (6.66)	2 (10.53)	4 (30.77)	
Left colon	4 (13.33)	5 (29.41)	10 (33.33)	1 (5.26)	2 (15.38)	
Rectum	20 (66.67)	10 (58.83)	18 (60.01)	16 (84.21)	7 (53.85)	
Tumor differentiation, n (%)						0.123
High	1(3.33)	4(23.53)	2(6.67)	1(5.26)	1(7.69)	
Middle	21(70.00)	12(70.59)	26(86.67)	13(68.42)	10(76.92)	
Low	8(26.67)	1(5.88)	2(6.66)	5(26.32)	2(15.39)	
TNM stage, n (%)						0.081
I	4(13.34)	4(23.53)	1(3.33)	4(21.05)	0(0)	
II	8(26.67)	10(58.82)	16(53.34)	6(31.58)	6(46.15)	
III	16(53.33)	3(17.65)	9(30.00)	9(47.37)	2(15.39)	
IV	2(6.66)	0(0)	4(13.33)	0(0)	5(38.46)	
Metastasis, n (%)						0.081
Yes	18(63.33)	3(23.52)	13(46.67)	9(52.63)	7(61.54)	
No	12(36.67)	14(76.48)	17(53.33)	10(47.37)	6(38.46)	
Residence, n (%)						0.792
Urban	13 (43.33)	9 (52.94)	15 (50.00)	11 (57.89)	7 (53.84)	
Rural	17 (56.67)	8 (47.06)	15 (50.00)	8 (42.11)	6 (46.16)	

a: χ^2 test.
Abbreviation: DHS: Damp-Heat Syndrome. SPOS:Stasis and Poison Obstruction Syndrome. SDQSS: Spleen Deficient Qi Stagnation Syndrome. LKYDS: Liver and Kidney Yin Deficiency Syndrome. QBDS: Qi and Blood Deficiency Syndrome.

sample on average, and 85,110 Effective Tags were obtained on average after quality control, with an average number of 68,506 valid quality control data and an effective quality control rate of 80.03 % (Table S1).

3.3. Clustering analysis of gut microbiota OTU in CRC patients with different TCM syndromes

The number of OTUs clustering in all samples included in this study was a total of 5,802, and the number and corresponding proportion of species at each level after species annotation using the database were: Kingdom level (n = 3, 96.69 %), Phylum level (n = 61, 85.90 %), Class level (n = 106, 83.88 %), Order level (n = 234, 76.47 %), Family level (n = 360, 63.84 %), Genus (Genus) level (n = 726, 39.95 %), and Species level (n = 463, 9.07 %). Due to the limitation of 16S rRNA gene sequencing technology, the number and corresponding proportion of species at the Species level were significantly reduced, so the subsequent discussion was mainly carried out at the phylum level and genus level. The clustering results of OTUs showed that the SDQSS group had more unique OTUs and the SPOS group had the least among the five groups of samples (Fig. 2. A).

3.4. Alpha diversity analysis of gut microbiota in CRC patients with different TCM syndromes

Rarefaction curve results showed that OTU numbers were significantly separated among the five groups when the sequencing depth was 50,000 (Fig. 2. B). Rank abundance curve showed a smooth decline in the curve, suggesting good species richness and evenness for all samples in this study (Fig. 2. C). In the Alpha diversity index analysis, when all data were normalized to 50501, we found that the Alpha diversity index showed significant differences between different TCM syndromes. The Shannon index was highest in the gut microbiota of SDQSS patients and lowest in the gut microbiota of LKYDS patients (p = 0.003). The ACE index was highest in the intestinal microbiota of SDQSS patients, but lowest in the intestinal microbiota of SPOS patients (p = 0.010). The PD whole tree index was highest in the gut microbiota of SDQSS patients, but lowest in the gut microbiota of SPOS patients (p = 0.017). Overall, among patients with different TCM syndromes, SDQSS patients had the highest gut microbiota biodiversity, and LKYDS patients and SPOS patients had relatively low gut microbiota biodiversity (Fig. 2. D-F).

3.5. Beta diversity analysis of gut microbiota in CRC patients with different TCM syndromes

In this study, the contribution value of PCo1 was 31.86 % and that of PCo2 was 5.62 % in principal co-ordinates analysis (Fig. 3. A). The diversity differences of gut microbiota between the two groups of different TCM syndromes was investigated using the analysis of differences between Adonis groups. The results showed that there were significant differences between the two groups (p < 0.05) except QBDS-LKYDS (p = 0.702), QBDS-DHS (p = 0.133), and SDQSS-DHS (p = 0.260).
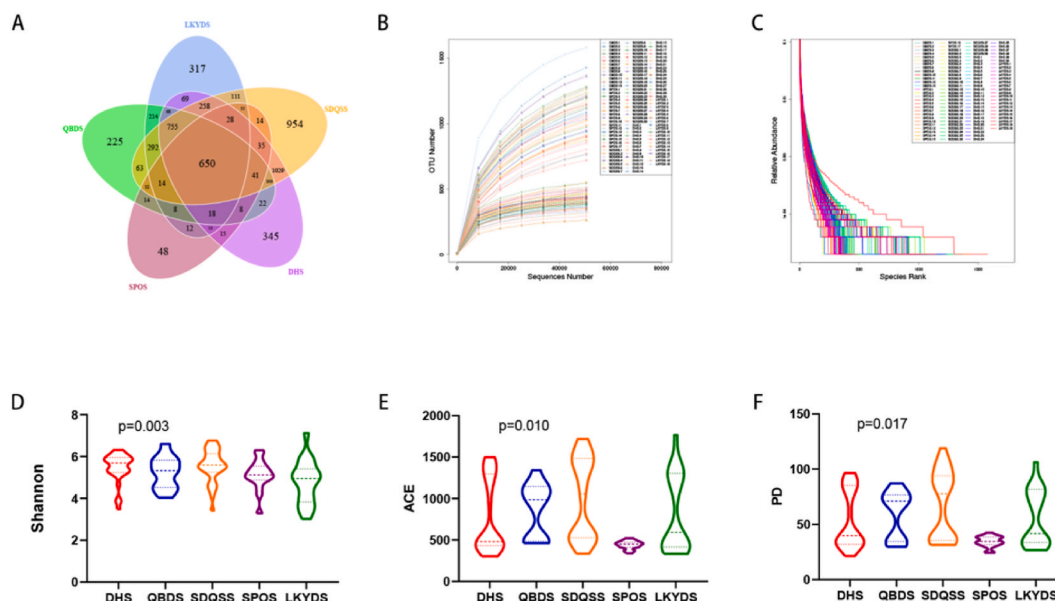

Fig. 2. Analysis of Gut microbiota in CRC Patients with Different TCM Syndromes. (A) OTU Clustering Result Plot. (B) Rarefaction Curve in Alpha Diversity Analysis. (C) Rank Abundance Curve in Alpha Diversity Analysis. (D–F) Alpha Diversity Correlation Index Analysis (Kruskal-Wallis H test, p < 0.05).
Abbreviation: DHS: Damp-Heat Syndrome. SPOS:Stasis and Poison Obstruction Syndrome. SDQSS: Spleen Deficient Qi Stagnation Syndrome. LKYDS: Liver and Kidney Yin Deficiency Syndrome. QBDS: Qi and Blood Deficiency Syndrome.
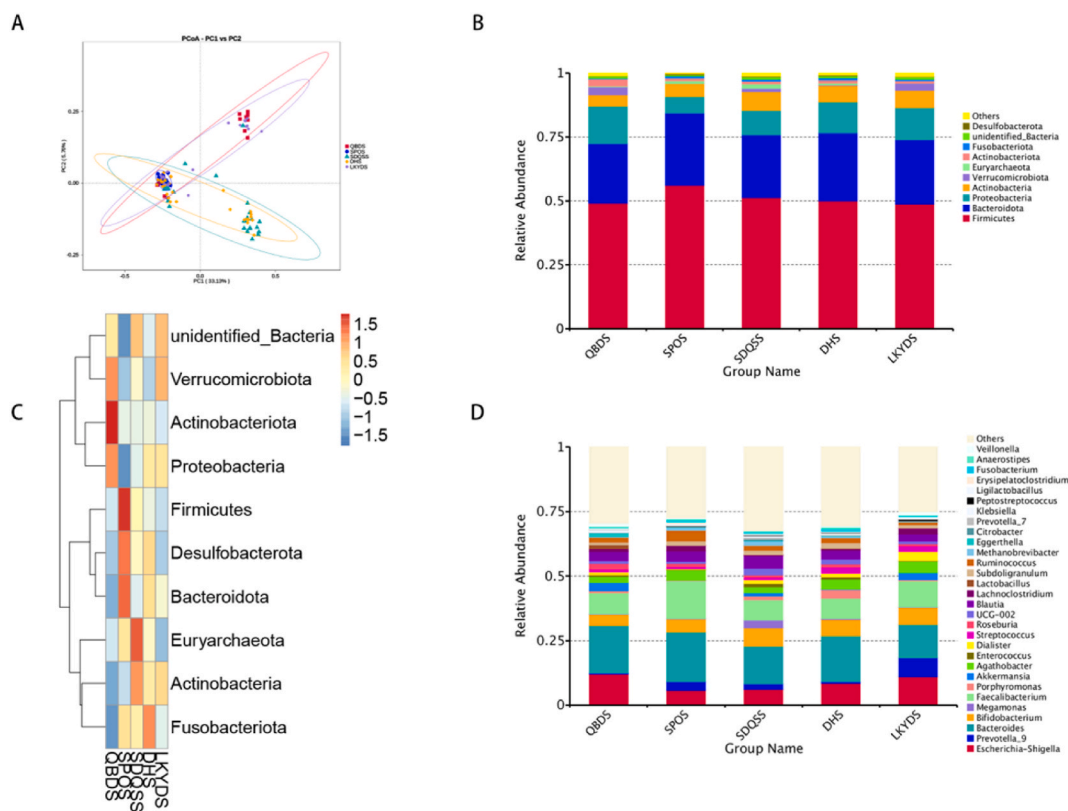
Fig. 3. Distribution of Gut microbiota in CRC Patients with Different TCM Syndromes. (A) Beta Diversity Analysis Results Plot. (B) Top 10 gut microbes in microbiota abundance at phylum level. (C) Abundance heat map of top 10 gut microbes at phylum level in gut microbiota of CRC patients with different TCM syndromes. (D) Top 30 gut microbes in microbiota abundance at genus level.

Abbreviation: DHS: Damp-Heat Syndrome. SPOS:Stasis and Poison Obstruction Syndrome. SDQSS: Spleen Deficient Qi Stagnation Syndrome. LKYDS: Liver and Kidney Yin Deficiency Syndrome. QBDS: Qi and Blood Deficiency Syndrome.

3.6. Distribution of gut microbiota at phylum and genus level in CRC patients with different TCM syndromes

The researchers collected all microbiota data in five different groups of TCM syndromes and summarized the top 10 gut microbes in microbiota abundance at the phylum level and the top 30 gut microbes in microbiota abundance at the genus level. At the phylum level, the dominant microbiota was generally consistent between the five groups, *Firmicutes*, *Bacteroidota*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobiota* accounted for more than 95 % of each group (Fig. 3 B–C). At the genus level, we obtained similar results to those at the gut microbiota phylum level in CRC patients with different TCM syndromes, and there were significant differences in the proportion of species composition between gut microbiota in CRC patients with different TCM syndromes (Fig. 3. D).*Bacteroides* and *Ruminococcus*, for example, were the most abundant in the SPOS group, accounting for 19 % and 4 %, and the least abundant in the LKYDS group, accounting for 12 % and 1 %.*Escherichia-Shigella* was the most abundant in the QBDS group, accounting for 12 %, but the least abundant in the SPOS group, accounting for 5 % (Fig. 4. A). Next, we further performed Kruskal-Wallis H test on the gut microbiota in the top 30 relative abundances at the genus level and found that 14 genera, *Prevotella9*, *Porphyromonas, Akkermansia, Enterococcus, Dialister, UCG-002, Lachnotidium, Lactobacillus, Ruminococcus, Eggerthella, Citrobacter, Pevotella7, Peptostreptococcus*, and *Veillonella*, were incompletely distributed in the five syndrome groups. For example, *Akkermansia* and *Veillonella* had high to low relative abundance of microbiota in the QBDS, LKYDS, SDQSS, DHS, SPOS groups. *Ruminococcus* was distributed from low to high relative abundance of microbiota in the QBDS, LKYDS, SDQSS, DHS, and SPOS groups (Table 2).

3.7. Analysis of enterotypes of gut microbiota at genus level in CRC patients with different TCM syndromes

In Beta diversity analysis of gut microbiota in CRC patients with different TCM syndromes, we observed that patients with QBDS/LKYDS had significantly different gut microbiota distribution in DHS/SDQSS. Therefore, we further performed enterotypes analysis on the genus-level gut microbiota of CRC patients with different TCM syndromes and determined the optimal number of classifications as five enterotypes according to the CH index (Fig. 5 A–B), which were named after the dominant bacteria among them, respectively. Among them, patients in the SDQSS group were more distributed in the *Megamonas* enterotypes, patients in the LKYDS group were more distributed in the *Prevotella9* enterotypes, patients in the DHS group were more distributed in the *Porphyromonas* enterotypes,
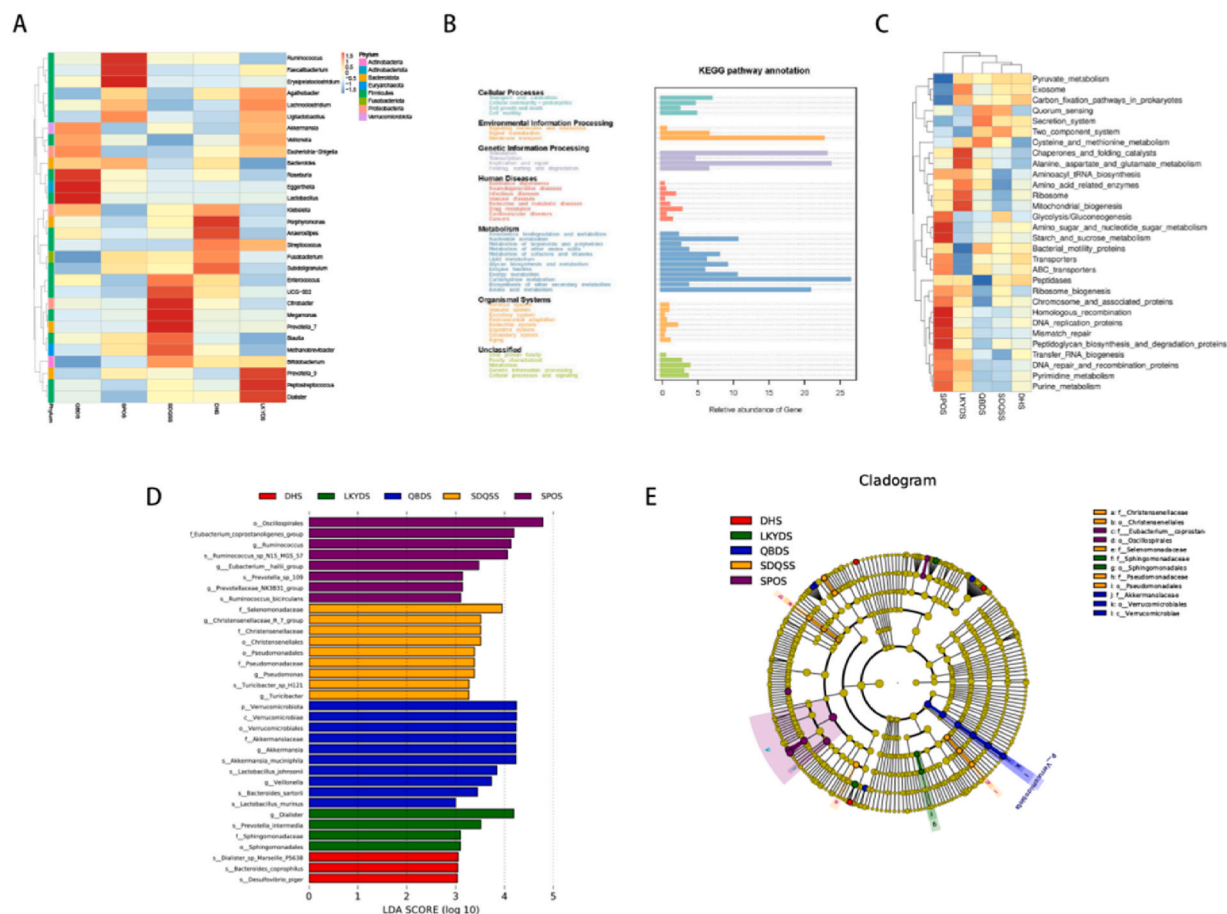
Fig. 4. Difference Analysis of Gut Microbiota in CRC Patients with Different TCM Syndromes. (A) Abundance heat map of top 30 gut microbes at genus level in gut microbiota of CRC patients with different TCM syndromes. (B) Predicting the biological function of gut microbiota using KEGG database. (C) Heat map of relevant metabolic pathways enriched by gut microbiota in CRC patients with different TCM syndromes. (D) Histogram of gut microbiota LEfSe analysis in CRC patients with different TCM syndromes. (E) Evolutionary branch diagram of LEfSe analysis of gut microbiota in CRC patients with different TCM syndromes (Only phylum to family microbiota are shown in this figure).
Abbreviation: DHS: Damp-Heat Syndrome. SPOS:Stasis and Poison Obstruction Syndrome. SDQSS: Spleen Deficient Qi Stagnation Syndrome. LKYDS: Liver and Kidney Yin Deficiency Syndrome. QBDS: Qi and Blood Deficiency Syndrome.

patients in the SPOS group were more distributed in the *Bacteroides* enterotypes, and patients in the QBDS group were more distributed in the *Escherichia-Shigella* enterotypes (Fig. 5 C-D).

3.8. Prediction of gut microbiota function in CRC patients with different TCM syndromes

In order to explore the biological function of gut microbiota in different TCM syndromes, the KEGG database and SILVA SSU Ref NR database were used to predict the biological function of gut microbiota in five groups of CRC patients. It was found that the gut microbiota of each group was predicted to be associated with seven types of biometabolic pathways, with the metabolism pathway being the most significant (45.60 %), followed by the genetic information processing pathway (24.28 %) and environmental information processing pathway (12.46 %), etc (Fig. 4. B). Next, the functional prediction pathways of the top 30 relative abundances of the second and third tier of KEGG metabolism in each group were analyzed, and a total of 44 metabolic pathways were found to be enriched at the second tier of KEGG metabolism, mainly carbohydrate metabolism (26.23 %), amino acid metabolism (20.59 %), nucleotide metabolism (10.35 %), and energy metabolism (10.27 %). A total of 390 metabolic pathways were found to be enriched at the third level of KEGG metabolism, for example, Amino sugar and nucleotide sugar metabolism, Starch and sucrose metabolism, Homologous recombination, DNA replication proteins, and Peptidoglycan biosynthesis and degradation proteins were enriched in the SPOS group, and pathways such as Chaperones and folding catalysts, and Mitochondrial biogenesis were enriched in the LKYDS group (Fig. 4. C). The results showed that although the metabolic pathways ranked in the top 30 in abundance were basically the same among the groups, there were still differences in the high-abundance metabolic pathways between different TCM syndromes, even if these differences were not statistically significant.

Table 2

Distribution differences of gut microbiota at genus level in CRC patients with different TCM syndromes.

Gut Microbiota	DHS (n = 30)	SPOS (n = 17)	SDQSS (n = 30)	LKYDS (n = 19)	QBDS (n = 13)	H	P
Escherichia-Shigella	0.085 ± 0.138	0.058 ± 0.124	0.061 ± 0.103	0.109 ± 0.172	0.121 ± 0.137	4.029	0.402
Prevotella_9	0.005 ± 0.006	0.034 ± 0.055	0.022 ± 0.057	0.075 ± 0.186	0.003 ± 0.004	10.089	0.039
Bacteroides	0.178 ± 0.128	0.195 ± 0.129	0.144 ± 0.128	0.129 ± 0.116	0.185 ± 0.126	5.785	0.216
Bifidobacterium	0.062 ± 0.070	0.051 ± 0.080	0.072 ± 0.113	0.066 ± 0.100	0.042 ± 0.052	3.109	0.540
Megamonas	0.003 ± 0.014	0.002 ± 0.006	0.031 ± 0.105	0.002 ± 0.005	0.000 ± 0.001	9.304	0.054
Faecalibacterium	0.079 ± 0.060	0.146 ± 0.106	0.077 ± 0.062	0.101 ± 0.118	0.083 ± 0.068	7.288	0.121
Porphyromonas	0.033 ± 0.083	0.001 ± 0.002	0.014 ± 0.034	0.004 ± 0.008	0.015 ± 0.049	31.103	0.000
Akkermansia	0.003 ± 0.006	0.001 ± 0.001	0.013 ± 0.031	0.028 ± 0.079	0.031 ± 0.092	14.498	0.006
Agathobacter	0.036 ± 0.035	0.040 ± 0.054	0.024 ± 0.021	0.044 ± 0.071	0.022 ± 0.019	4.803	0.308
Enterococcus	0.009 ± 0.039	0.003 ± 0.002	0.012 ± 0.056	0.003 ± 0.010	0.007 ± 0.019	10.281	0.036
Dialister	0.014 ± 0.029	0.003 ± 0.005	0.016 ± 0.040	0.034 ± 0.073	0.010 ± 0.017	17.771	0.001
Streptococcus	0.024 ± 0.048	0.008 ± 0.017	0.008 ± 0.015	0.023 ± 0.062	0.010 ± 0.014	7.553	0.109
Roseburia	0.012 ± 0.018	0.013 ± 0.016	0.010 ± 0.010	0.008 ± 0.011	0.023 ± 0.060	2.472	0.650
UCG-002	0.019 ± 0.019	0.009 ± 0.010	0.027 ± 0.040	0.012 ± 0.014	0.011 ± 0.016	14.200	0.007
Blautia	0.033 ± 0.022	0.040 ± 0.024	0.048 ± 0.046	0.028 ± 0.023	0.036 ± 0.036	7.336	0.119
Lachnoclostridium	0.008 ± 0.009	0.020 ± 0.029	0.005 ± 0.003	0.022 ± 0.042	0.011 ± 0.020	23.697	0.000
Lactobacillus	0.001 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.001 ± 0.001	0.014 ± 0.048	17.775	0.001
Subdoligranulum	0.021 ± 0.027	0.018 ± 0.012	0.016 ± 0.030	0.013 ± 0.011	0.012 ± 0.013	5.351	0.253
Ruminococcus	0.020 ± 0.020	0.041 ± 0.039	0.019 ± 0.019	0.010 ± 0.009	0.018 ± 0.011	19.979	0.001
Methanobrevibacter	0.007 ± 0.028	0.010 ± 0.040	0.017 ± 0.039	0.001 ± 0.001	0.003 ± 0.012	7.771	0.100
Eggerthella	0.001 ± 0.001	0.003 ± 0.002	0.000 ± 0.000	0.001 ± 0.001	0.015 ± 0.043	32.818	0.000
Citrobacter	0.002 ± 0.005	0.001 ± 0.002	0.007 ± 0.026	0.000 ± 0.000	0.002 ± 0.003	18.972	0.001
Prevotella_7	0.001 ± 0.001	0.000 ± 0.000	0.007 ± 0.026	0.001 ± 0.001	0.000 ± 0.000	13.496	0.009
Klebsiella	0.010 ± 0.027	0.002 ± 0.003	0.006 ± 0.016	0.003 ± 0.004	0.010 ± 0.020	9.245	0.055
Peptostreptococcus	0.002 ± 0.002	0.000 ± 0.001	0.003 ± 0.003	0.007 ± 0.026	0.002 ± 0.002	21.434	0.000
Ligilactobacillus	0.003 ± 0.004	0.009 ± 0.028	0.004 ± 0.008	0.009 ± 0.020	0.003 ± 0.006	2.038	0.729
Erysipelatoclostridium	0.001 ± 0.001	0.008 ± 0.028	0.001 ± 0.001	0.001 ± 0.002	0.002 ± 0.003	6.839	0.145
Fusobacterium	0.010 ± 020	0.008 ± 0.027	0.008 ± 0.015	0.006 ± 0.011	0.003 ± 0.003	2.581	0.630
Anaerostipes	0.006 ± 0.019	0.003 ± 0.002	0.003 ± 0.005	0.002 ± 0.002	0.004 ± 0.005	4.240	0.375
Veillonella	0.005 ± 0.010	0.006 ± 0.011	0.001 ± 0.001	0.011 ± 0.029	0.012 ± 0.017	18.707	0.001

Kruskal-Wallis H rank sum test.

Abbreviation: DHS: Damp-Heat Syndrome. SPOS:Stasis and Poison Obstruction Syndrome. SDQSS: Spleen Deficient Qi Stagnation Syndrome. LKYDS: Liver and Kidney Yin Deficiency Syndrome. QBDS: Qi and Blood Deficiency Syndrome.

3.9. LEfSe analysis of gut microbiota in CRC patients with different TCM syndromes

Finally, in order to screen representative microbiota of different TCM syndromes and analyze their related action pathways affecting TCM syndromes, the screening value of linear regression analysis value (LDA Score) was set as 3 to select the signature microbiota in different TCM syndromes for gut microbiota LEfSe analysis and further screen the differential representative microbiota corresponding to five different TCM syndromes. The results showed that there were three signature microbiotas in the DHS group, which were *Dialister* sp *Marseille P5638*, *Bacteroides-coprophilus*, and *Desulfovibrio piger*, among them, the representative microbiota was *Dialister* sp *Marseille P5638* (LDA = 3.05, p < 0.001). There were eight signature microbiotas in the SPOS group, which were *Oscillospirales*, *Eubacterium coprostanoligenes group*, *Ruminococcus*, *Ruminococcus* sp *N15 MGS 57*, *Eubacterium hallii group*, *Ruminococcus bicirculans*, *Prebicaceae NK3B31 group*, and *Prevotella* sp *109*, among them, the representative microbiota was *Oscillospirales* (LDA = 4.78, p = 0.029). There were nine signature microbiotas in the SDQSS group, which were *Christensenellales*, *Christensenellaceae*, *Christensenellaceae R-7 group*, *Turicibacter*, *Turicibacter* sp *H121*, *Pseudomonadales*, *Pseudomonadaceae*, *Selenomonadaceae*, and *Pseudomonas*, among them, the representative microbiota was *Selenomonadaceae* (LDA = 3.94, p = 0.003). There were four signature microbiotas in the LKYDS group, which were *Sphingomonadales*, *Sphingomonadaceae*, *Dialister*, and *Prevotella intermedia*, among them, the representative microbiota was *Dialister* (LDA = 4.19, p = 0.001). There are 10 signature microbiotas in the QBDS group, which were *Verrucomicrobiota*, *Verrucomicrobiae*, *Verrucomicrobiales*, *Akkermansiaceae*, *Akkermansia*, *Akkermansia muciniphila*, *Lactobacillus johnsonii*, *Lactobacillus murinus*, *Veillonella*, and *Bacteroides sartorii*, among them, the representative microbiota was *Akkermansia muciniphila* (LDA = 4.23, p = 0.006) (Fig. 4. D, E). These results indicated that there were significant differences in representative microbiota between different TCM syndromes in CRC patients, reflecting the material basis regarding gut microbiota between different TCM syndromes.

4. Discussion

TCM believes that the syndromes in the early stage of CRC are mainly excess syndromes such as DHS and SPOS, and the syndrome of intermingled deficiency & excess such as SDQSS are mainly in the middle stage, while the deficiency syndromes such as LKYDS and QBDS are mainly in the late stage [16]. Therefore, in this study, 109 stool samples from preoperative CRC patients were collected to investigate the differences in gut microbiota among CRC patients with different TCM syndromes by 16S rRNA gene sequencing and seek representative microbiota of CRC patients with different TCM syndromes in order to assist TCM physicians in more accurate
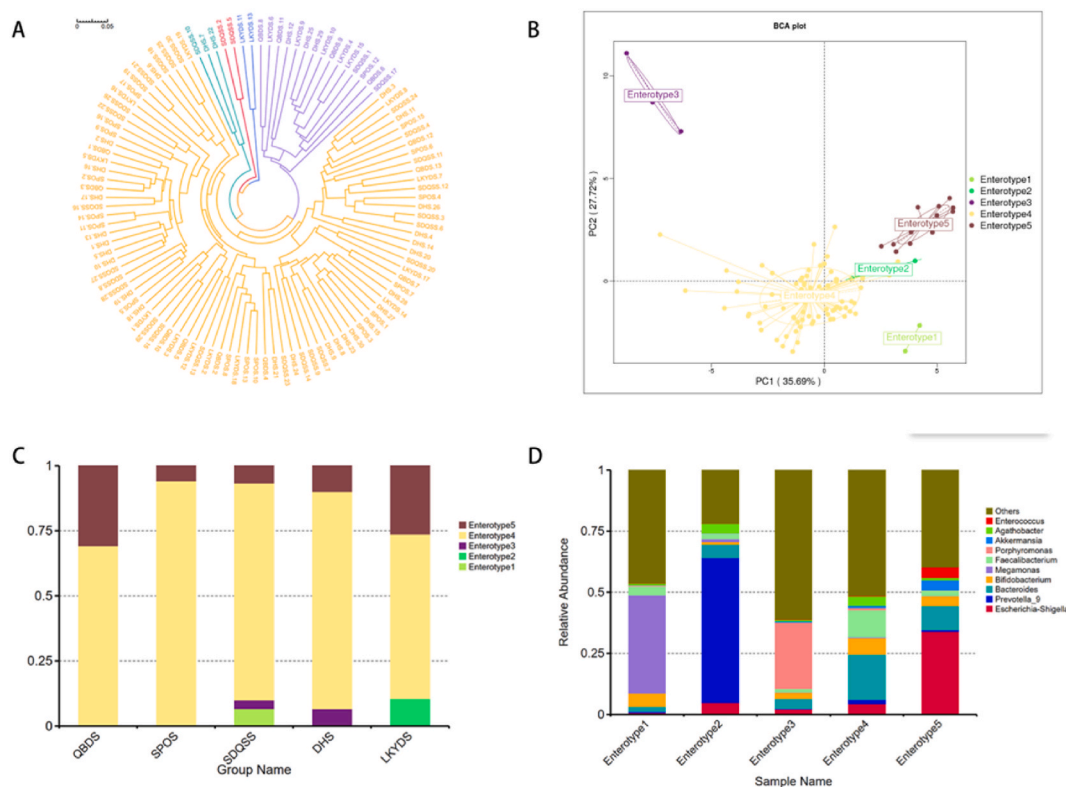
Fig. 5. Analysis of Enterotypes of Gut Microbiota at Genus Level in CRC Patients with Different TCM Syndromes. (A) Clustering of individual samples in enterotypes. (B) Enterotypes based BCA analysis. (C) Distribution of each phenotype in enterotypes. (D) Histogram of relative abundance of top10 species in each enterotype.

Abbreviation: DHS: Damp-Heat Syndrome. SPOS:Stasis and Poison Obstruction Syndrome. SDQSS: Spleen Deficient Qi Stagnation Syndrome. LKYDS: Liver and Kidney Yin Deficiency Syndrome. QBDS: Qi and Blood Deficiency Syndrome.

clinical syndrome differentiation using representative gut microbiota of different TCM syndromes.

It was found that among the 109 included preoperative CRC patients, there were more male patients than female patients, and the gender of patients among the five groups of TCM syndromes was statistically significant, which may be related to the higher incidence of CRC in Chinese males than in females. According to the report on cancer incidence and mortality in China released in 2022, the incidence of CRC in China is $33.68/10^5$ in males and $25.13/10^5$ in females [17], and the incidence in males is much higher than that in females. The male CRC patients included in this study were more than the female CRC patients, which is consistent with the reported incidence.

The results of OTU cluster analysis of gut microbiota in CRC patients with different TCM syndromes in this study revealed that the SDQSS group had more unique OTUs and the SPOS group had the least in the five groups of samples. Alpha diversity analysis of gut microbiota revealed that gut microbiota biodiversity was highest in SDQSS patients and lowest in LKYDS and SPOS patients. The results showed that SDQSS patients had more gut microflora among the five TCM syndromes. This is not consistent with the findings of Xu et al. [18], who had only 5 samples in the SDQSS group in their study. Therefore, differences between sample sizes may be an important cause of inconsistent results. At the phylum level, the top 10 gut microbes in microbiota abundance were basically the same, with *Firmicutes, Bacteroidota, Proteobacteria, Actinobacteria,* and *Verrucomicrobiota* occupying more than 95 % of each group. However, the abundance content of each microbiota in different TCM syndromes showed significant differences. Similarly, we observed consistent results at the genus level. These results indicate that in the gut microbiota of CRC patients, the microbiota species will not change with changes in TCM syndromes, but the relative abundance of microbiota in the gut microbiota will change in different TCM syndromes. A series of related studies have emerged in recent years to influence CRC tumor development by increasing or decreasing the abundance of gut microbiota. For example, Yang et al. [19] found that the High-fat diet drives colorectal tumorigenesis through inducing gut microbial dysbiosis. Yachida et al. [20] found in a large cohort metagenomics study of CRC patients that the relative abundance of *Fusobacterium nucleatum, Atopobium parvulum*, and *Actinomyces odontolyticus* was significantly increased in CRC patients. In CRC, up-regulation of *Fusobacterium nucleatum* abundance was found to be able to influence the CRC tumor microenvironment [21]. According to the characteristics of gut microbiota, more and more researchers have proposed many new therapeutic strategies for reversing gut microbiota, including antibiotics and fecal microbiota transplantation, etc [22].

Next, we further performed enterotypes analysis based on the results of Beta diversity analysis of gut microbiota in CRC patients with different TCM syndromes. enterotypes are described as clusters with dense samples in a multidimensional space composed of

communities, which are not influenced by factors such as age, sex, and geographic location [23]. Among the dominant genera of enterotypes corresponding to excess syndromes(DHS and SPOS), *Porphyromonas* is common in oral infections and root canal infections, and *Porphyromonas gingivalis* is associated with tumor common molecular subtypes, which are closely related to colorectal cancer. Gingival proteases of *P. gingivalis* can invade colorectal cancer cells and promote their proliferation by activating the MAPK/ERK signaling pathway in colorectal cancer cells, and their peptidoglycan can promote PD-L1 expression up-regulation in colon cancer cells [24]. *Bacteroides* is one of the intestinal endosymbionts involved in many important metabolic activities in the human colon, including fermentation of carbohydrates, utilization of nitrogen-containing substances, and biotransformation of bile acids and other steroids [25]. *Enterotoxigenic Bacteroides fragilis*, a target of tight junctions in intestinal cells, leads to E-cadherin cleavage. As reactive oxygen species increase, it increases intestinal permeability and triggers chronic intestinal inflammation through NF-κB signaling and tissue damage, ultimately leading to the development of CRC. Syndrome of intermingled deficiency & excess (SDQSS) corresponds to the dominant enterotypes of microbiota. *Megamonas* has not been reported as a dominant genus in gut microbiota studies in European American subjects. However, it has been confirmed in relevant studies of Chinese individuals, indicating that this genus may be a microbiota characteristic of Asian populations [26]. And the relative abundance of this genus was negatively correlated with colorectal polyp incidence [27]. Deficiency syndrome (QBDS and LKYDS) corresponds to the dominant enterotypes of microbiota. *Escherichia-Shigella* was confirmed to be an important pathogen causing bacillary dysentery [28]. *Prevotella9* has been demonstrated to have a positive correlation with the prognosis of CRC [29]. High-fiber diet can increase the abundance of *Prevotella9* and promote the intestinal abundance of *Lactobacillus* and *Bifidobacterium* without affecting the diversity of gut microbiota [30]. This result confirms that different TCM syndromes in CRC patients may be related to enterotypes, but its specific mechanism remains to be further explored. Secondly, the biological function of gut microbiota in different TCM syndromes was investigated through the KEGG database. The results showed that the biological function of gut microbiota in five TCM syndromes was mainly related to metabolic pathways. It was further analyzed and found that the gut microbiota of different TCM syndromes was involved in different metabolic processes under metabolic pathways. Among the five TCM syndromes, the metabolic process of gut microbiota was most enriched in the SPOS group and least in the DHS group.

In order to screen representative microbiota of different TCM syndromes, the signature microbiota of different TCM syndromes (LDA Score = 3, p < 0.05) was screened, and LEfSe analysis of gut microbiota was performed. The results showed that QBDS had the most signature microbiotas and LKYDS had the least signature microbiotas among the five groups. The representative microbiota for each TCM syndrome was further screened, where the representative microbiota in the DHS group was *Dialister* sp *Marseille P5638 (DSM-P5638)*. *DSM-P5638* is a Gram-negative anaerobe bacterium (*family Veillonellaceae, phylum Firmicutes*). *DSM-P5638* was found to be enriched in the gut microbiota of patients with liver and gallbladder tumors refractory to PD-L1 therapy. *DSM-P5638* was associated with increased bile acids, increased bilirubin, and decreased liver function, demonstrating a possible link to energy metabolism [31]. The representative microbiota of the SPOS group was *Oscillospirales* (*phylum Firmicutes*), which is one of the important bacteria producing butyrate [32]. It is worth noting that two syndromes, DHS and SPOS, are considered excess syndrome in TCM and are usually in the early stages of the disease. *DSM-P5638* served as a representative microbiota for DHS, and the effects of *DSM-P5638* on bilirubin and liver function were consistent with previous findings by our team. It has been confirmed in the past that bilirubin levels and liver function are significantly higher in excess syndrome patients than in patients with deficiency syndrome and syndrome of intermingled deficiency & excess [14]. Butyrate is a short-chain fatty acid that is generally considered to be associated with promoting the proliferation of colonic epithelial cells and inhibiting the growth of tumor cells, and butyrate is not only able to promote the expression of histone deacetylase (HDAC) [33], but also can increase the infiltration of CD8+ T cells into the tumor and improve the body's immune function [34].

The representative flora of the SDQSS group is *Selenomonadaceae*, which is a Gram-negative anaerobic bacterium (*phylum Proteobacteria*) and functions to produce pro-inflammatory factors [35]. *Proteobacteria* are present in low abundance in the gut microbiota of normal humans, and intestinal inflammation or antibiotic treatment increases oxygenation of colonic epithelial cells, thereby destroying anaerobic organisms and driving dysregulated expansion of facultative anaerobic *Proteobacteria* through aerobic respiration [36]. Related studies have found that the relative abundance of *Proteobacteria* is significantly increased when the body suffers from intestinal inflammation or CRC disease [37,38], causing gut microbiota imbalance. The representative flora in the LKYDS group was *Dialister* (*phylum Firmicutes*). *Dialister* is a Gram-negative anaerobic bacillus, and Tito et al. [39] found that the relative abundance of *Dialister* in the bacterial community was significantly increased in patients with intestinal inflammation relative to healthy individuals. In terms of neoplastic disease, *Dialister* was reported to have significantly elevated relative abundance in cervical cancer [40], gastric cancer [41], and CRC, and it was found that elevated relative abundance of *Dialister* may be associated with lymph node metastasis in CRC [42].

The representative microbiota of the QBDS group was *Akkermansia muciniphila* (*A. muciniphila*). *A. muciniphila* is a Gram-negative anaerobe bacterium (*phylum Verrucomicrobiota*). It has been shown that *A. muciniphila* supplementation can promote activation of the NF-κB/NLRP3 signaling pathway and infiltration of M1 macrophages and increase anti-tumor immune responses [43,44]. Shi et al. [45] found that oral *A. muciniphila* combined with IL-2 treatment significantly enhanced the anti-tumor therapeutic effect of IL-2, and *A. muciniphila* could protect intestinal barrier function and maintain mucosal homeostasis under systemic IL-2 treatment. From the perspective of TCM understanding CRC, QBDS and LKYDS belong to the category of TCM deficiency syndrome. Compared with excess syndrome patients, their immune function is lower [46]. Interestingly, in this study, we found that *A. muciniphila* showed high abundance in both QBDS and LKYDS TCM syndromes, combined with *A. muciniphila* having the effects of improving the body's immune function and enhancing the body's anti-tumor response, and speculated that the reason for its increased abundance may be related to the body's immune compensatory mechanism.

Syndrome differentiation is not only the primary principle in TCM diagnosis and treatment of diseases, but also the premise of

individualized TCM treatment. Because the gut microbiota is in close contact with CRC tumors, and there is a dynamic balance between the human body and its gut microbiota that is extremely similar to the understanding of diseases in TCM. Therefore, an exploratory study of gut microbiota between different TCM syndromes was conducted. Among the five groups of gut microbiota of CRC patients with different TCM syndromes, the diversity and complexity of intestinal microbiota were the highest in CRC patients in the SDQSS group and the lowest in the SPOS group. Analysis at the phylum and genus level of gut microbiota revealed that the species of microbiota in CRC patients with different TCM syndromes were basically the same, but the abundance of their dominant microbiota showed significant differences. Finally, the representative microbiota of different TCM syndromes was further screened, and it was believed that this difference in microbiota abundance and the representative microbiota between each syndrome may help TCM physicians to more accurately discern the TCM syndromes of CRC patients.

5. Conclusion

The aim of this study was to investigate the differences in gut microbiota among different TCM syndromes in CRC patients and to understand the material basis between different TCM syndromes from the microbiota level. In this study, the abundance differences of gut microbiota in CRC patients with different TCM syndromes was introduced at the phylum level and genus level, and the representative microbiota of different TCM syndromes was further screened, which were significantly increased in abundance in different TCM syndromes and are expected to provide a reference for accurate dialectical of TCM physicians in clinical practice. Overall, this study preliminarily confirmed that there is a certain link between TCM syndromes and gut microbiota in CRC patients, and these findings may provide a theoretical basis for the objective analysis of TCM syndromes in CRC.

Ethics statement

This research plan has been reviewed and approved by the 940th Hospital of Joint Logistics Support Force of Chinese People's Liberation Army Clinical Research Ethics Committee of the hospital (No.2020KYLL075).

Data availability statement

Data associated with this study were not stored in publicly available repositories. All data in this study included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Gui Ming-bin: Writing – original draft. **Wang Ya-nan:** Writing – original draft, Writing – review & editing. **Xue Yong-ting:** Data curation. **Zou Min:** Data curation. **Tu Hao:** Data curation. **Qu Lian-ping:** Data curation. **Gao Feng:** Project administration, Supervision, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21057.

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