Role of Traditional Chinese Medicine Syndrome Type, Gut Microbiome, and Host Immunity in Predicting Early and Advanced Stage Colorectal Cancer

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Abstract

Objective: To investigate the role of Traditional Chinese Medicine (TCM) syndrome type, gut microbiome distribution, and host immunity function in predicting the early and advanced clinical stages of colorectal cancer (CRC). **Methods:** A cross-sectional case-control study was performed which included 48 early stage and 48 advanced patients with CRC enrolled from March 2018 to December 2020. 16S rRNA gene sequencing was performed to analyze the gut microbiomes of the patients, while T and B lymphocyte subsets in peripheral blood were assessed using flow cytometry. TCM syndrome type was measured using the spleen deficiency syndrome (SDS) scale. **Results:** The abundance levels of *Prevotella, Escherichia-Shigella*, and *Faecalibacterium* in the gut microbiota were significantly increased in the advanced group, while *Bacteroides* was significantly decreased. *Phascolarctobacterium* was detectable only in the early metaphase group, whereas *Alistipes* was detectable only in the advanced group. The lymphocyte ($P = .006$), T helper cell (TH) ($P = .002$), cytotoxic T cell (TC) ($P = .003$), double positive T cell (DPT) ($P = .02$), and total T counts ($P = .001$) were significantly higher in the early metaphase group than in the advanced metaphase group. Compared with patients with early stage CRC, the advanced group had a higher SDS score. After adjusting for clinical stage, Spearman's correlation analysis showed interactions among gut microbiome abundance, T cell level, and SDS score. Multivariate logistic analysis showed that after controlling for the SDS score, abundance of *Alistipes* and *Faecalibacterium*, and double negative T cell (DNT) level, DPT was significantly associated with a lower risk of advanced-stage disease (hazard ratio, 0.918; $P = .022$). **Conclusion:** Our study suggested associations between clinical stage, SDS, gut microbiota, and T lymphocytes, which provided insights for a potential prediction model for the disease progression of CRC.

Keywords

clinical stage, colorectal cancer, gut microbiome, T cell immunity, traditional Chinese medicine syndrome

Introduction

Colorectal cancer (CRC) is a major cause of cancer-related deaths and one of the most common malignancies worldwide.[1] It results from a multifactorial interaction including individual genetic background,[2,3] environmental factors,[4] and lifestyle.[5] Long-term survival analysis showed that T stage, N stage, original tumor site, genotype, and lifestyle may influence the risk of recurrence and metastasis in patients with early stage CRC.[6-8] In recent years, evidence of the relationship between CRC prognosis and gut microbiome has increased rapidly.[9-12] A growing body of research tends toward the development of predictive models for CRC development and disease progression by integrating potential biomarkers and verifying them in prospective studies.[13,14]

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According to Traditional Chinese Medicine (TCM) theory, spleen-deficiency syndrome (SDS) is one of the key syndrome types in the development of CRC. Previously, our research team published a validated TCM-SDS scale[15] that included 5 items of patient-reported SDS symptoms. Additionally, we found that the SDS score was significantly higher among patients with advanced CRC than among those with early stage CRC. Since patients with SDS mainly report symptoms of fullness, diarrhea, and fatigue, it has been hypothesized that SDS may be related to dysfunction of the gut microbiome and host immunity regulation. Both clinical and *in vivo* studies have shown that TCM herbs that target SDS could modulate the gut microbiome of participants and animals and regulate T cell immunity.[16] However, no direct evidence exists to support the correlation between SDS, the gut microbiota, and host immunity in patients with CRC.

Increasing evidence indicates that the gut microbiota is an important contributor in colorectal cancer initiation, progression, and metastasis.[17] Intestinal bacteria can generate a distinct immune microenvironment by inducing abnormal immunological responses in colorectal tissue, compromising the intestinal epithelial barrier, and producing tumor-causing toxins that can act on intestinal epithelial cells and stimulate cell proliferation.[18] Furthermore, carcinogenic metabolites, reactive oxygen species, and other free radicals are produced by these toxins, all of which cause DNA damage in the host cells and encourage mutations. These mechanisms together promote the spread of CRC.[19] Additionally, CRC progression can be linked to functional impairment of host immunity. Alterations in the gut microbiota due to SDS may result in an imbalance of T cell and B cell subpopulations, triggering the release of pro-inflammatory and anti-inflammatory cytokines. Therefore, whether the interaction between SDS, the gut microbiome, and host immunity could predict the prognosis of CRC requires further investigation.

In this study, we conducted a cross-sectional study including 96 patients with CRC; 16S rRNA gene sequencing was performed to analyze the gut microbiota of these patients. The microbial spectrum of patients with CRC from early metaphase to advanced stages was elucidated, and the severity score of SDS and absolute number of T and B lymphocytes in different clinical stages were identified. Furthermore, we investigated the predictive role of gut microbiota, SDS score, and T and B lymphocytes in different clinical stages of CRC using a multivariate regression model.

Methods

Study Design and Population

A cross-sectional study was conducted at Xiyuan Hospital of the China Academy of Chinese Medical Sciences and Beijing Cancer Hospital between March 2018 and December 2020. This was a prospective, observational study to investigate the SDS properties of patients with CRC and diversity of microbiota at different clinical stages by convenience sampling. Demographic and lifestyle information was collected at baseline from the National Key R&D Program of China and The National Natural Science Foundation of China. Some of this information included sex, age, body mass index (BMI), primary tumor site, tumor type, and TCM-SDS.

Participants needed to meet the American Joint Committee on Cancer (AJCC) histological diagnosis standards and exhibit clear TNM staging by clinical imaging or pathology. Patients with stages I, II, and III CRC were divided into early metaphase groups. Patients with stage IV CRC were placed in the advanced group. Eligible patients were 18 to 80 years old, consented to participate, and had good compliance. The exclusion criteria for the study were as follows: history of previous malignancy; under active treatments including chemotherapy, targeted therapy, or immunotherapy; clinically relevant cardiovascular and/or cerebrovascular disease; active hepatitis; severe abnormalities in liver/renal function tests. And use of antibiotics or probiotics 1 month prior to the enrollment was also an exclusion criterion. The research protocol was approved by the ethics committee at each site and all patients provided written informed consent (certificate no. 2018XLA048-2; 2016XLA122-4).

TCM SDS Scale Analysis

The diagnostic criteria were determined according to the standardization of commonly-seen malignant tumors. The identification of SDS was conducted using a patient-reported outcome (PRO), which was developed by our team to evaluate SDS. The Traditional Chinese Medicine Spleen Deficiency Syndrome Scale (TCM-SDS) is a PRO-TCM-SDS scale for CRC that has shown repeated reliability and consistency.[15] To calculate the scale scores, we standardized the scale as follows: standardization scores/10 = actual scores/(5 × numbers of items).

The scale comprises 5 items: *I feel a loss of appetite, I feel abdominal fullness, I feel my arms and legs lack strength, I feel short of breath when talking, and my stool is formless.* Each item was rated on a five-point Likert-type scale (1 = never, 2 = occasionally, 3 = sometimes, 4 = often, and 5 = always).

Stool Sample Collection and Microbial Diversity Analysis

We collected patients' fecal samples in special tubes containing DNA stabilizer, which were sent to Majorbio (Shanghai, China) for analysis. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff

using UPARSE (version 7.1, http://drive5.com/uparse/) with a novel "greedy" algorithm which performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database, using a confidence threshold of 70%.

T and B Lymphocyte-Subset Analysis

The distribution of T and B lymphocyte subsets in peripheral blood samples was assessed using flow cytometry (Navios; Beckman Colter, USA) and subdivided according to the clinical stage of the disease. These included lymphocytes, $CD3^+$ (total T cells, T), $CD4^+$ $CD8^+$ (double-positive cells, DPT), $CD4^+$ (helper T cells, Th), $CD8^+$ (cytotoxic T cells, Tc), $CD3^+(CD16 + CD56)^+$(natural killer T cells, NKT), $CD3^-($ $CD16 + CD56)^+$(natural killer cells, NK), $CD3^+$ $CD19^-$ (total B cells, B), and fluorescently labeled monoclonal antibodies (Beckman Colter) of the BD Multitest 6-Color TBNK Reagent, namely CD3-FITC, CD25-PE, and HLA-DR-PC5.

Sample Size

The sample size was calculated based on a minimum of 10 to 15 times the total number of TCM-SDS items. There were 5 items on this scale, so the sample size was 75. Assuming a sample loss rate of 10% to 15%, the final sample size was defined as 100.

Statistical Analysis

The total TCM-SDS scale scores were calculated by summing the 5 individual items and normalizing them to a value between 0 and 10. Higher scores signify a greater SDS burden. Data analyses and preparation of graphs were performed using SPSS v26.0 and GraphPad Prism v9.0. Patient characteristics are expressed as numbers and percentages, or as medians and interquartile ranges (IQR) for non-normally distributed data or means and standard deviations (SD) for normally distributed data. Pearson's chi-square or Fisher's exact test was used for comparing categorical variables between the groups. A multivariate logistic-regression model was used to analyze the independent impact factors on the early or advanced stages of CRC. Statistical significance was set at $P < .05$.

Results

In this study, 96 patients with CRC from 2 centers (March 2018 to December 2020) were included in the analysis. Figure 1 illustrates the flow of the study.

Characteristics of Patient at Different Clinical Stages

The demographic information and clinical characteristics of all the included individuals are shown in Table 1. The majority of patients in the advanced group (45.85%) had rectal cancer. There were no significant differences in demographics, primary tumor site, or tumor type among the groups.

Association Between the TCM-SDS Score and Clinical Stage

Patients with advanced CRC had significantly higher SDS scores than patients with early metaphase CRC (3.71 ± 1.97 vs 4.11 ± 1.39; $P = .032$) (Figure 2).

Analysis of T and B Lymphocyte Subsets of Different Clinical Stages

In total, peripheral blood samples from 53 patients were finally collected. The analysis revealed that the lymphocyte count ($P = .006$), TH count ($P = .002$), TC count ($P = .003$), DPT count ($P = .02$), and T count ($P = .001$) were significantly higher in the early metaphase group than in the advanced group. Compared to the advanced group, the absolute number of T lymphocyte subsets was higher in patients with early metaphase. The outcomes for all the above results are shown in Figure 3.

Gut Microbial Profiles of Patients with Different Clinical Stage CRC

Among 96 patients who had been enrolled in this study, 83 stool samples were finally collected and 1 352 900 high-quality 16S rRNA gene sequence reads were obtained. A total of 3218 OTUs were identified. The stool microbiota estimated in the rarefaction analysis indicated clear asymptotes, indicating that the sequencing depth per sample covered most of the diversity and reached a saturated sample capacity.

Microbial Richness and Diversity Analysis of Patients at Different Clinical Stages

In the alpha diversity analysis, Sobs and the Chao indices were used to describe community richness and diversity. According to alpha diversity indices, community richness tended to be significantly higher in patients with advanced stage CRC than those with early metaphase CRC (Sobs index: 410.93 ± 170.14 vs 304.92 ± 149.6, $P = .0042$; Chao index: 567.95 ± 220.74 vs 424.75 ± 201.84, $P = .0033$). Moreover, in the beta diversity analysis, the principal
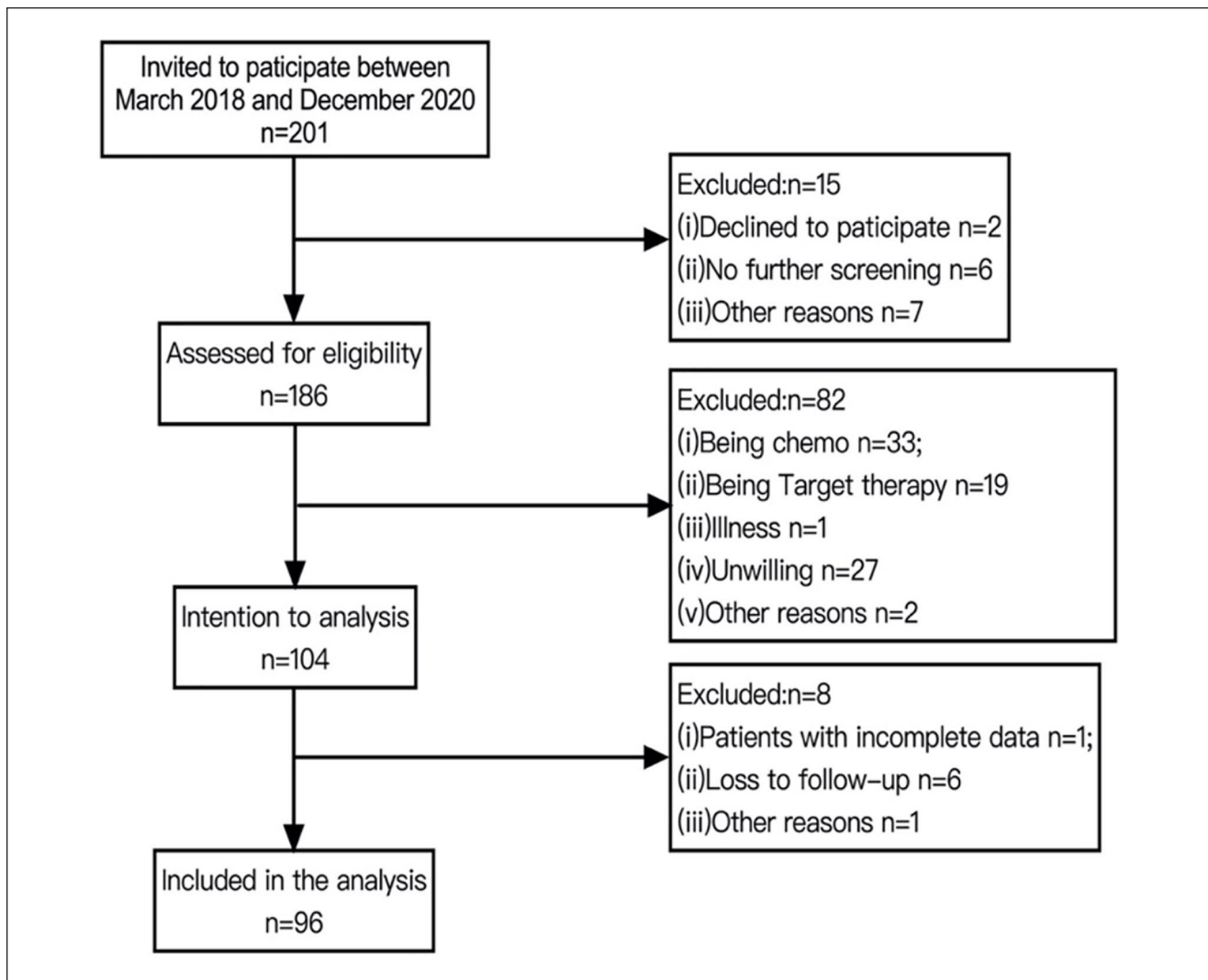
Figure 1. Flow diagram for the study.

Table 1. Baseline Characteristics of the Study Population.

	Early metaphase (n = 48)	Advanced (n = 48)	P value
Demographics/anthropometric			
Age (mean ± SD)	62.83 ± 7.22	62.65 ± 11.37	.92
Male/female	29/19	35/13	.19
BMI (kg/m²)	22.83 ± 2.59	22.44 ± 3.08	.50
Primary tumor site			.46
Rectum	17 (35.42%)	22 (45.83%)	
Right colon	16 (33.33%)	11 (22.92%)	
Left colon	15 (31.25%)	15 (31.25%)	
Tumor type			.66
Adenocarcinoma	39 (81.25%)	36 (75%)	
Mucinous adenocarcinoma	6 (12.5%)	5 (10.42%)	
Signet-ring cell carcinoma	2 (4.17%)	0 (0%)	
Neuroendocrine carcinoma	1 (2.08%)	0 (0%)	

Abbreviations: BMI, body mass index; SD, standard deviation.
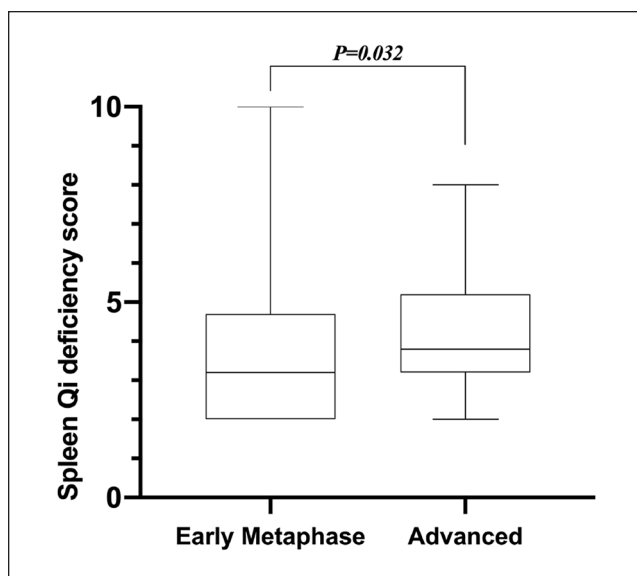
Figure 2. SDS score of different CRC clinical stages.

coordinates analysis (PCoA) revealed that the gut microbiota in the early metaphase group was significantly different from that in the advanced metaphase group ($P = .001$). The gut microbial taxa changed progressively with the progression of CRC from early metaphase to advanced stages. Moreover, analysis of similarity (ANOSIM) confirmed that the groups were significantly different (Figure 4).

Microbiota Composition Changes Between Different Clinical Stages

At the genus level, the gut microbiota was dominated by *Bacteroides*, *Prevotella*, *Escherichia-Shigella*, and *Faecalibacterium* at different disease stages. After further analysis at the genus level, the more abundant species in the early metaphase group were primarily from *Bacteroides* (34.47%). In contrast, abundant species in the advanced group were mainly from *Prevotella* (8.21%), *Escherichia-Shigella* (6.70%), and *Faecalibacterium* (7.23%). *Phascolarctobacterium* (3.21%) was detectable only in the early metaphase group, whereas *Alistipes* (3.57%) was detected only in the advanced group (Figure 5). This indicates that the composition of the gut microbiota may have changed during the progression of CRC.

Specific Gut Microbial Signature in Different Clinical Stages

Detailed analyses revealed certain unique microbial taxa in the early metaphase and advanced groups. At the genus level, we observed 4 bacterial taxa with different abundances between the 2 groups. *Bacteroides* and *Phascolarctobacterium* were highly enriched in the early metaphase group. Conversely, *Faecalibacterium* and *Alistipes* were significantly enriched in the advanced group (Figure 6).

Correlation Between Gut Microbiota in Different Clinical Stages

The association between SDS, T and B lymphocyte subsets, and the gut microbiota, was explored by performing a Spearman's correlation analysis. A significant positive correlation between the abundance of *Blautia* and Tc cells, Th cells, T cells, and lymphocytes were observed when adjusting for clinical stage. In contrast, the abundance of *Christensenellaceae_R-7_group* and *norank_f__norank_o_ Clostridia_UCG-014* was negatively associated with Tc cells, Th cells, T cells, and total lymphocyte counts. Moreover, a significantly significant positive association was observed between the abundance of *Lachnospiraceae_ NK4A136_group* and TCM-SDS scores, and an increase in the severity of SDS was positively associated with the abundance of *Lachnospiraceae_NK4A136_group* (Figure 7).

Multivariate Logistic Regression Analysis

After controlling for the SDS score, abundance of *Alistipes* and *Faecalibacterium*, and DNT level, DPT was significantly associated with a lower risk of advanced stage CRC (Hazard Ratio, 0.918; $P = .022$). Higher SDS scores and abundance of *Alistipes* were associated with a higher risk of advanced stage disease; however, this was not significant ($P = .069$ and 0.078, respectively) (Table 2).

Discussion

In this clinical observational study, the relationships among CRC clinical stage, TCM-SDS level, gut microbiome, and T cell immunity were explored, as well as the feasibility of establishing a potential predictive model for CRC prognosis by utilizing the aforementioned factors. Through multivariate analysis, we found that DPT count was a significant independent risk factor of being in the advanced CRC, while a higher SDS score and enriched abundance of *Faecalibacterium* and *Alistipes* were related to a lower risk. Under the concept of precision medicine, developing a predictive model for disease prognosis and treatment effects has become a major focus in this field.[20,21] Existing predictive factors for CRC prognosis include genomic type and original tumor site.[22,23] The gut microbiome has been found to potentially impact the effect of immunotherapy in CRC.[24,25] The current study is the first to evaluate the predictive role of TCM syndrome differentiation, gut microbiome, and T cell immunity in the prognosis of CRC.
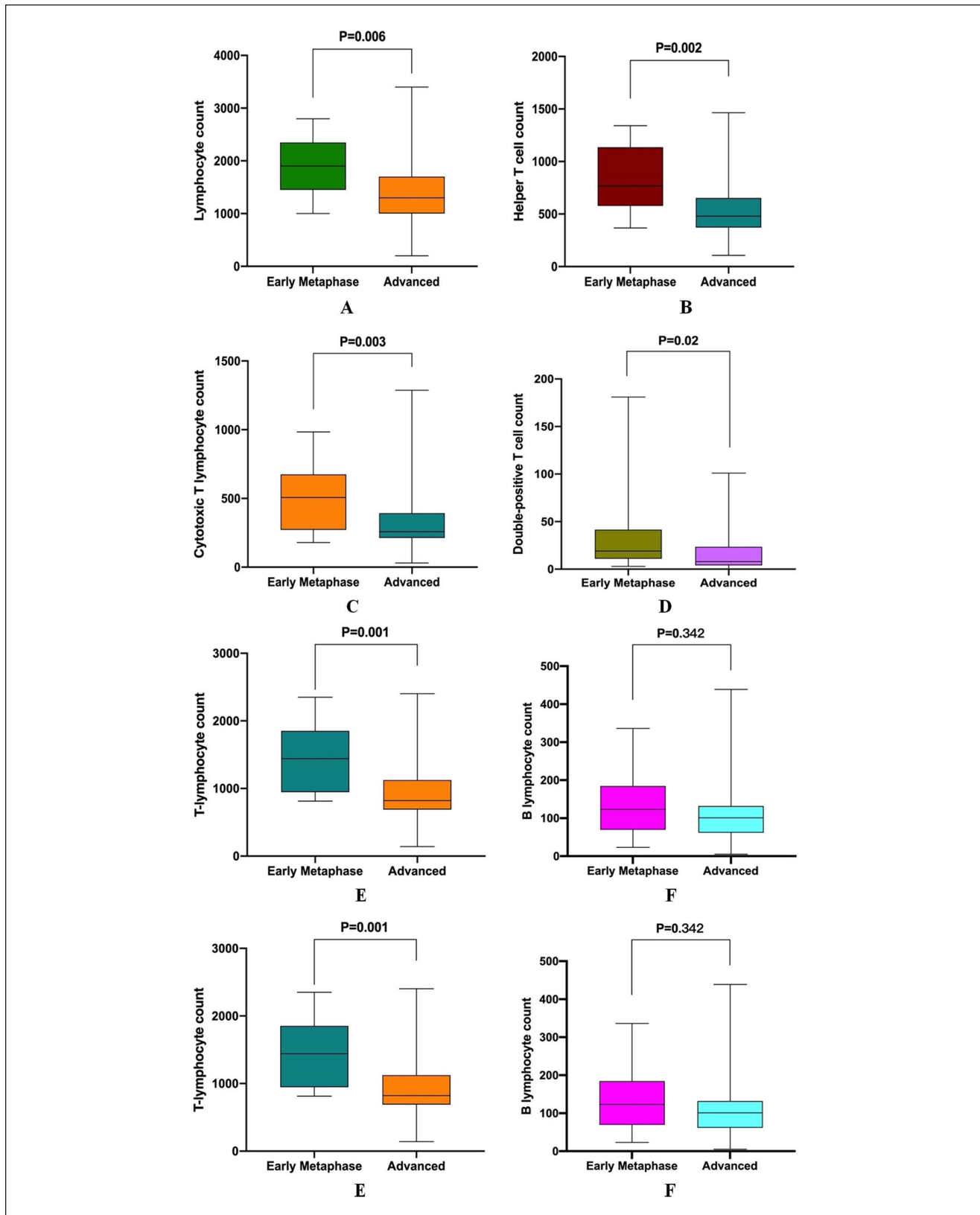
Figure 3. The TB lymphocyte subset analysis in different clinical stages. In part of (A), (B), (C), (D), (E), (F), (G), (H), the values of the data were shown with median ± quartiles (25,75).
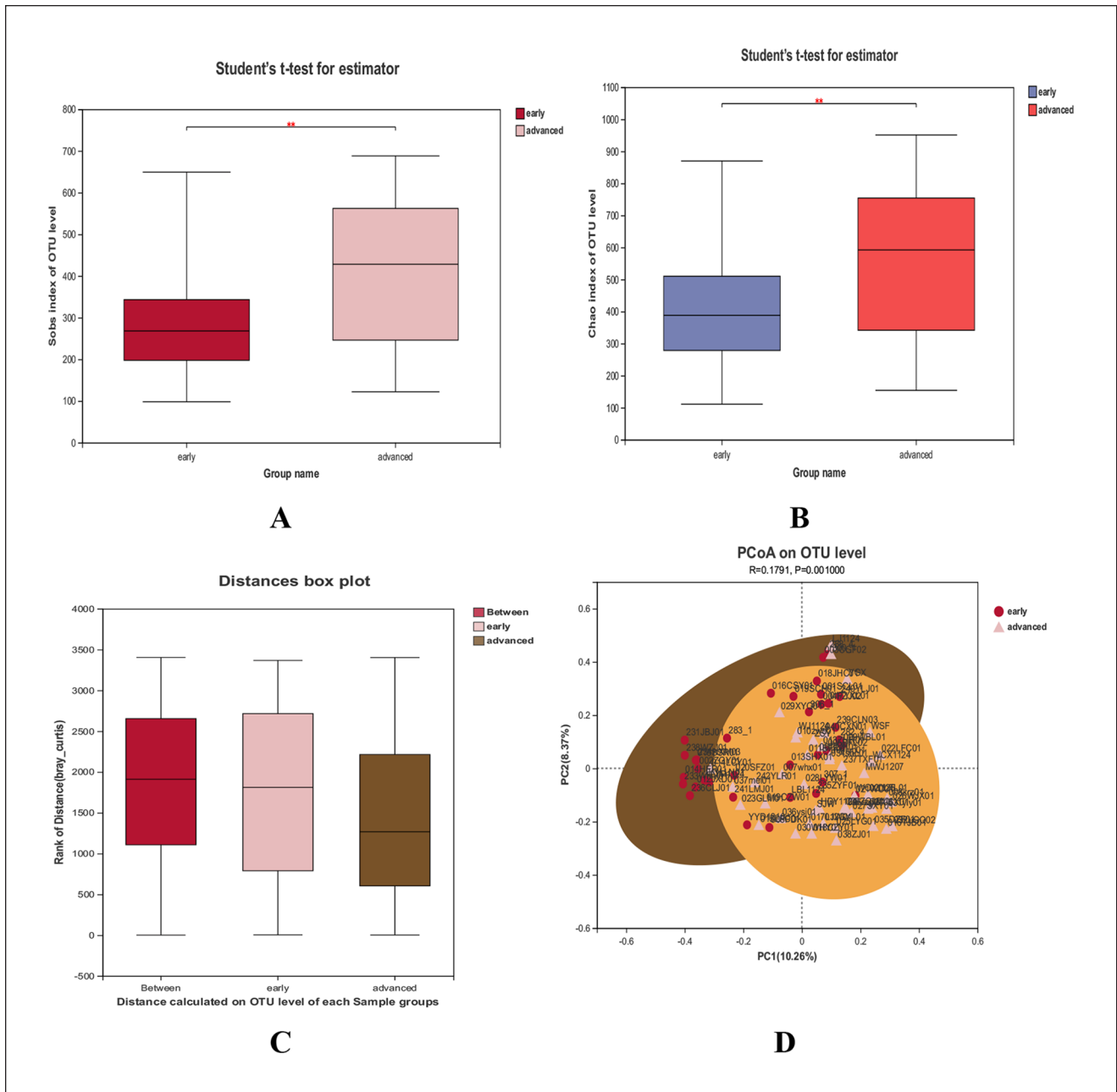
Figure 4. The gut microbiota alpha diversity and beta diversity analysis between different clinical stages. (A) Boxplot of the Student's-test for the Sobs index. (B) Boxplot of the Student's-test for the Chao index. (C) Boxplot of similarity analysis for identifying the differences between the 2 groups. (D) Principal coordinates analysis (PCoA) for the gut microbial community composition.

There are an increasing number of studies illustrating the micro-mechanism of TCM syndrome differentiation in CRC. An observational study showed that patients with CRC and SDS had decreased levels of CD3+, CD4+, and NK cells, as well as lower whole blood viscosity (WBV), plasma viscosity (PV), hematocrit (Hct), erythrocyte sedimentation rate (ESR), and plasma fibrinogen concentration (PFC). Different TCM syndrome types have also been found to be independently associated with CRC prognosis.[26] One study found that patients with CRC and *Pi-Xu ZHENG* (SDS) had higher wild-type TP53 and KDM6A mRNA levels in their blood samples.[27] Metabolomic analysis of patients with CRC and Qi deficiency showed increased metabolites involved in galactose and linolenic acid metabolism.[28] Wang et al[29] compared the gut microbiome distribution of healthy individuals and patients with CRC
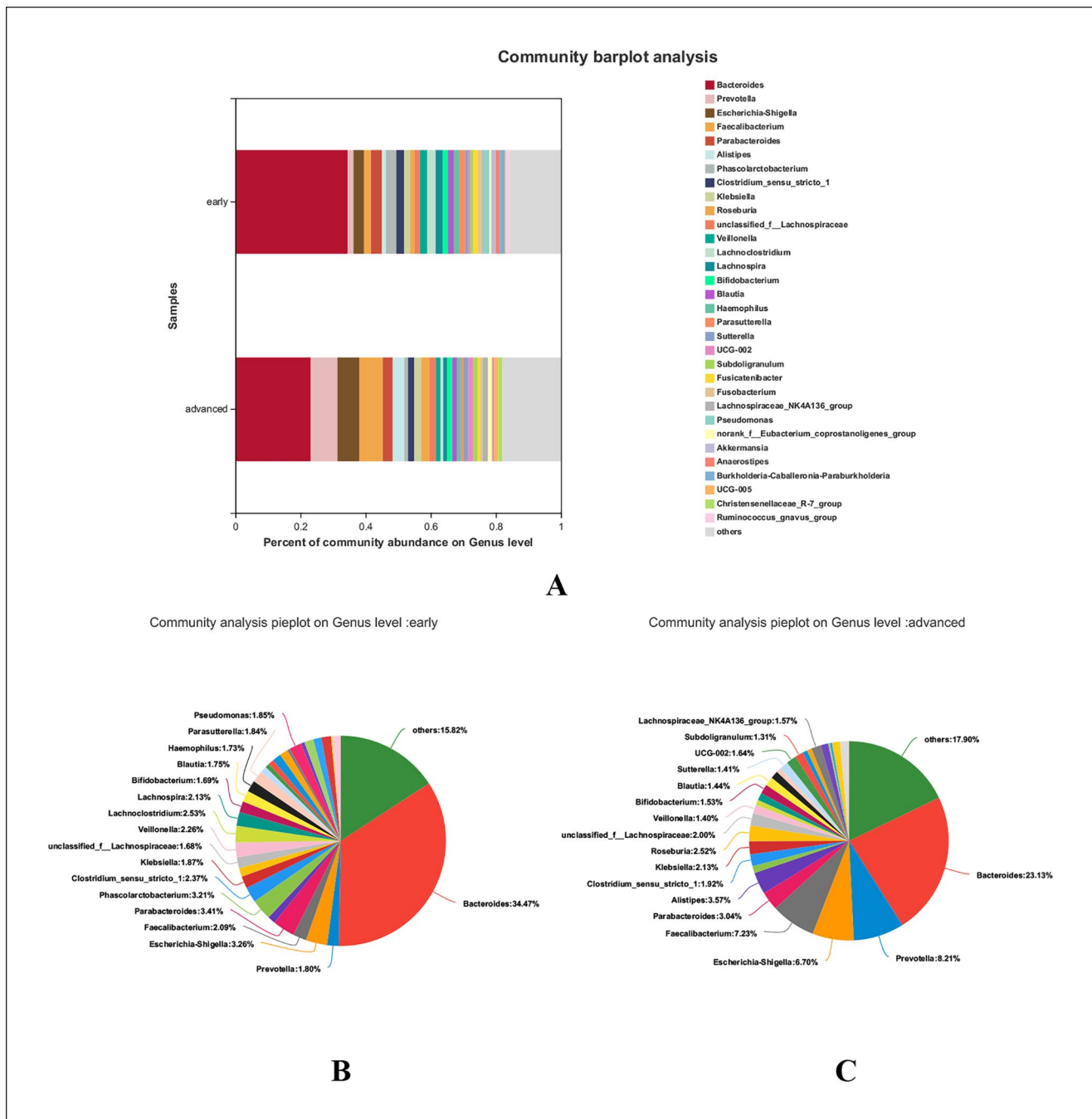
Figure 5. Comparison of relative abundance among each group Bar plots of the relative abundance of the main bacterial taxa at the genus level for the control the early metaphase group and the advanced group. (A) Bar plots of the abundance distribution of species at the level of genus. (B) Pie chart of community analysis on genus level in early metaphase group. (C) Community analysis pie plot on genus level in advanced group.

and Zheng-Qi-Kui-Xu (Qi deficiency) or Xie-Du-Yong-Sheng(excessive pathogen infection) and found that the abundance of *Lactobacillales* was enriched in the Zheng-Qi-Kui-Xu group. In the current study, a positive association was observed between the abundance of the gut bacteria *Lachnospiraceae_NK4A136_group* and severity of SDS. Although members of *Lachnospiraceae* are among the main producers of short-chain fatty acids, different taxa
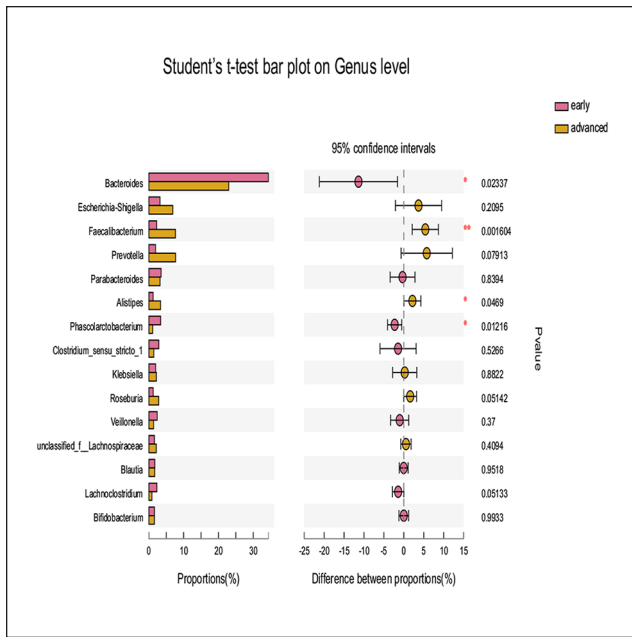
Figure 6. Differentiation analysis bar plot on genus level between different clinical stages.

of *Lachnospiraceae* are also associated with different intra- and extra-intestinal diseases,[30] which may partly explain our findings.

Patients with advanced stage CRC had lower abundance of *Bacteroides*, and increased levels of *Prevotella*, *Escherichia-Shigella*, and *Faecalibacterium* compared with those the early-stage group. The abundance of *Phascolarctobacterium* was exclusively enriched in the early stage group, and *Alistipes* was significantly enriched in the advanced group. *Phascolarctobacterium* is a short-chain fatty acid producer that also produces acetate and propionate.[31] *Phascolarctobacterium* has been positively correlated with the induction of colonic interferon-γ (IFN-γ)-expressing CD8 T cells, and colonization of mice with a consortium of 11 bacterial strains, including *Phascolarctobacterium faecium*; this has been shown to enhance both spontaneous and immune checkpoint blockade (ICB)-mediated anti-tumor activity by increasing CD8+ tumor-infiltrating lymphocytes producing IFN-γ in syngeneic tumor models.[32] *Alistipes* is a relatively new sub-branch genus of the *Bacteroidetes* phylum, which is frequently linked to persistent intestinal inflammation. *Alistipes* dysbiosis is either beneficial or harmful. *Alistipes* has been shown to be harmful in CRC cases, acting as a potential pathogen, which can lead to the fermentation of undigested proteins in the GI tract and generation of toxic compounds by bacteria. Another study showed that *Alistipes* was strongly positively correlated with an increased tumor burden.[33] Despite its pathogenic effects

in patients with CRC, *Alistipes* has been demonstrated to play a helpful role in cancer immunotherapy by altering the tumor microenvironment. One study found that when *Alistipes* abundance was reduced, optimal responses to cancer immunotherapy were reduced. Therefore, future research should further investigate the role of *Alistipes* as a prospective biomarker for CRC progression.

In the current study, we found that the absolute number of T lymphocyte subsets was relatively higher in the early metaphase group than in the advanced group, in which DPT was independently associated with a lower risk of developing advanced disease. Peripheral DPT lymphocytes are immunomodulators of the immune response. One study revealed that DPT was significantly decreased in patients with CRC compared with healthy individuals, and this decline was related to a lower abundance of *Faecalibacterium prausnitzii*.[34] Similarly, the multivariate analysis in our study showed that the abundance of *Faecalibacterium* was related to a higher risk of being in the advanced group. The current predictive model still needs to be improved by expanding the sample size and optimizing the design.

Our study has certain limitations. First, prognostic outcomes, including disease-free and overall survival, may be more appropriate for establishing a predictive model for CRC disease progression. Our research team planned to follow up with all the participants enrolled in the current study to further verify the predictive role of SDS, the gut microbiome, and T cell immunity on CRC survival outcomes. Additionally, although the current study excluded patients who were undergoing active cancer treatment, the differences in disease characteristics between early stage and advanced CRC could still be influenced by previous cancer treatments, comorbidities, and lifestyle factors. Future studies should control for all possible confounders in the inclusion criteria or through multivariate analysis methods. Machine learning is an innovative application for establishing a predictive model for CRC prognosis. This was a cross-sectional study that was susceptible to sampling bias and it was difficult to make a causal inference. Future research should include a longitudinal cohort study which predefines the TCM-SDS level, gut microbiome, and T cell immunity of patients with CRC at baseline and follows up with them to determine the survival outcomes after controlling for other confounding factors such as treatments, original tumor sites, and genomic type.

In conclusion, our study suggested that patients with advanced CRC had higher SDS scores, increased abundance of *Alistipes* and *Faecalibacterium* in their gut microbiota, and lower serum DNT levels compared with patients with early stage CRC. This provided insights into a potential prediction model for the disease progression of CRC.
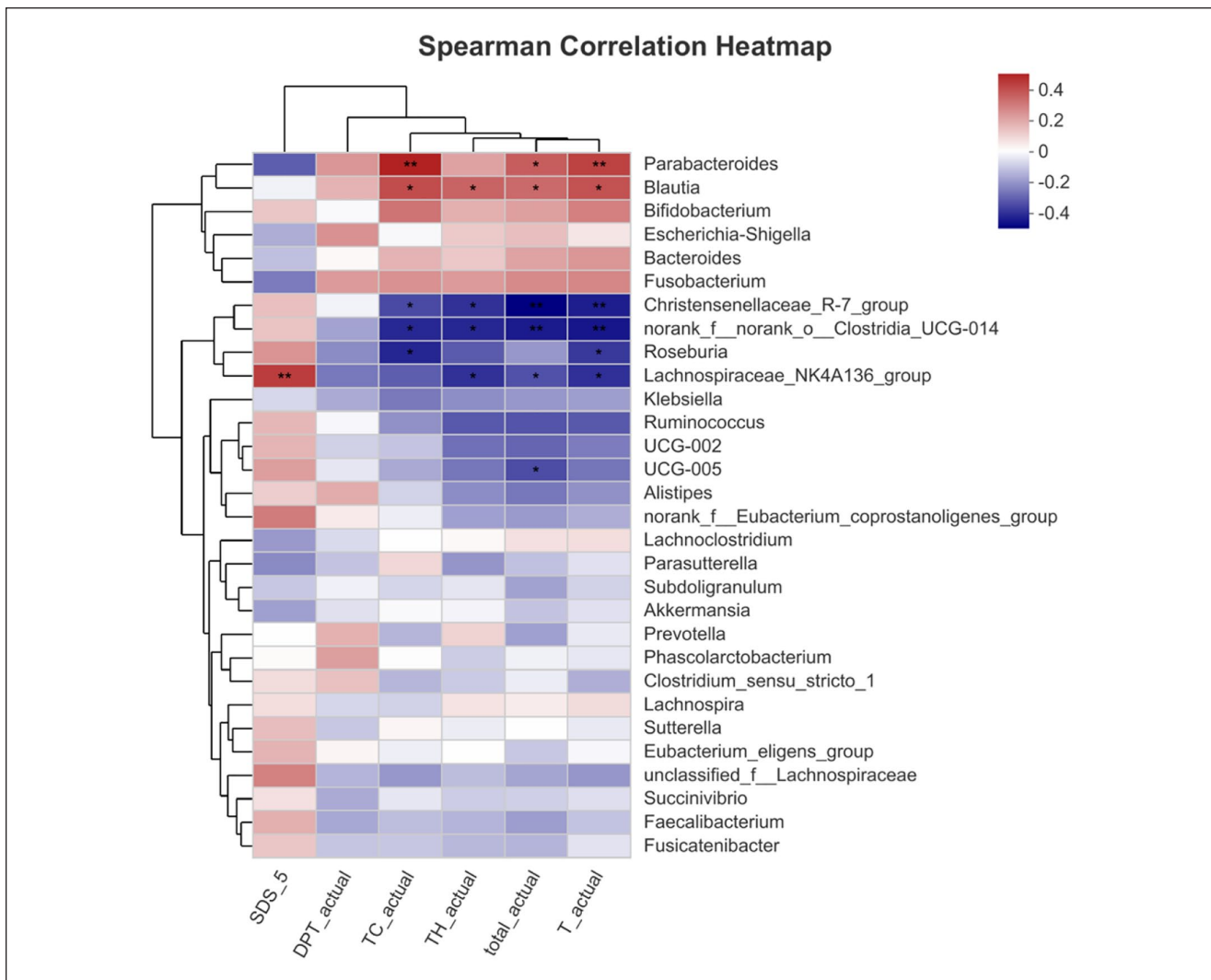
Figure 7. Association of the TB lymphocyte subsets and SDS with gut microbiota on genus level.

Table 2. Multivariate Regression Model on Risk Factors of Advanced CRC.

Factor	Hazard ratio[*]	95% CI	*P* value
SDS score	1.84	0.95, 3.54	.07
DPT	0.92	0.85, 0.99	.02
DNT	1.01	0.99, 1.03	.15
Alistipes	5.39×10^9	$0.079, 3.68 \times 10^{20}$	.08
Faecalibacterium	1.5×10^5	$0.01, 2.18 \times 10^{12}$	.16

Abbreviations: SDS, spleen deficiency syndrome; DNT, double-negative T cell; DPT, double-positive T cell; 95% CI, 95% confidential interval.
*Hazard ratio was the risk entering into the advanced CRC group compared with the early stage group.

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Declaration of Conflicting Interests

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