

Characterization of the Fecal Microbiota in Gastrointestinal Cancer Patients and Healthy Controls

Ningning Li

Peking Union Medical College Hospital

Chunmei Bai (✉ baichunmei1964@126.com)

Peking union medical college hospital <https://orcid.org/0000-0003-1333-9145>

Yuanzhi Guan

Chinese Academy of Medical Sciences and Peking Union Medical College Institute of Basic Medical Sciences

Lin Zhao

Peking Union Medical College Hospital

Yuping Ge

Peking Union Medical College Hospital

Xiaoyuan Li

Peking Union Medical College Hospital

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Abstract

Background The incidence and mortality of gastrointestinal tumors are high in China. Some studies suggested that the gut microbiota is also related to the occurrence and development of tumors. At present, there are no prospective studies based on the correlation between gastrointestinal tumors and gut microbiota in the Chinese population. The objective of this report is to characterize the fecal microbiota in patients with esophageal cancer, gastric cancer, and colorectal cancer and healthy controls.

Methods Patients with locally advanced or metastatic esophageal, gastric, and colorectal cancer were enrolled, and healthy people were included as controls. 16S rRNA sequencing was used to analyze the characteristics of fecal microbiota. PICRUST software was used for functional prediction.

Results Significant differences in the composition and abundance of fecal microbiota were identified between gastrointestinal cancer patients and healthy controls. The abundance of *F. prausnitzii*, *C. clostridioforme*, and *B. adolescents* in three different tumor groups was all significantly lower than in the control group ($P < 0.05$). The abundance of *R. gnavus*, *B. obeum*, and *R. faecis* in the gastric and colorectal cancer groups was significantly lower than in the control group ($P < 0.05$). The abundance of *Dorea* in the gastric cancer group was significantly lower than in the control group ($P < 0.05$). *B. fragilis*, *P. corpi*, and *E. coli* were overabundant in the different tumor groups compared with the control ($P < 0.05$). There were significant differences in functional metabolism and cell biological function between the tumor and control groups ($P < 0.05$). The feces of cancer patients with more advanced staging had higher abundance of *Prevotella* and fewer *Clostridium*.

Conclusions Patients with esophageal or gastric cancer had similar features of fecal bacteria as those with colorectal cancer. The metabolic function of fecal bacteria in the gastrointestinal cancer patients and the healthy controls were different.

Introduction

The incidence and mortality of gastrointestinal (GI) cancers have increased worldwide^[1]. In China, gastric cancer is the most common GI cancer and the second leading cause of cancer death. The incidence and mortality of esophageal and colorectal cancer are also in the top five. In 2015, there were 1533300 new cases of esophageal cancer, gastric cancer, and colorectal cancer in China, and 1064000 cases died^[2]. The mortality rate was as high as 69%.

The occurrence and development of GI tumors are the result of multiple factors. Changes in genes or epigenetics, immune status, diet and environmental factors, and infection of pathogenic microorganisms may be relevant^[3]. The human gut is a complex biological system. There are approximately 3.8×10^{13} microorganisms in the gut. They are mainly composed of bacteria, archaea, fungi, protozoa, and viruses. These microorganisms are involved in maintaining normal physiological function and regulating immunity and metabolism^[4, 5]. In recent decades, gut microbiota has been reported to be involved in

various diseases such as tumors^[6]. Based on the primary sites of GI tumors, gut microbiota may play a role in the occurrence and development of GI cancer.

To date, research on the association between microbiota and GI tumors has mainly focused on colorectal cancer. With the development of bioinformatics and high throughput sequencing, the mechanisms demonstrating that gut microbiota interacts with colorectal cancer by producing specific toxins^[7], affecting the signaling pathways^[8], and regulating immune response^[9] have been verified. Microbiological studies of esophageal cancer and gastric cancer mostly focused on the upper gastrointestinal microbiota. The microbiota in the digestive juice and tissues of the upper digestive tract were reported to be related to the pathogenesis of esophageal and gastric cancer^[10]. Some bacteria have been identified to have effects on the response of chemotherapy in colon cancer and lymphoma in mouse models^[11, 12]. Immunotherapy has been widely used in recent years. In a study by Sivan et al^[13] in 2018, 16S ribosomal RNA (rRNA) sequencing identified *Bifidobacterium* as associated with the anti-programmed cell death ligand-1 (PD-L1) effects. Other analyses using 16S rRNA sequencing conducted on fecal samples revealed a significant enrichment in the Ruminococcaceae family in responding melanoma patients undergoing anti-programmed cell death receptor-1 (PD-1) immunotherapy with enhanced anti-tumor immunity^[14]. Metagenomics of fecal samples of patients with non-small cell lung cancer and renal cell carcinoma demonstrated associations between *Akkermansia muciniphila* and the clinical responses of immunotherapy^[15].

Few studies have been conducted on the association between GI tumors and fecal microbiota in Asian populations. Based on the previous data on the role of the gut microbiota in other types of tumors, we hypothesized that the gut microbiota may be linked to the prevention or pathogenesis of different kinds of GI cancers. Therefore, we prospectively studied the fecal microbiota composition and functional prediction using 16S rRNA sequencing to profile the bacterial communities in patients with esophageal cancer, gastric cancer, and colorectal cancer and healthy controls. Our goals were to evaluate the characteristics of the fecal microbiota of GI tumors.

Materials And Methods

Study subjects and sampling

This study prospectively included 81 patients with GI cancers from April 2018 to April 2019 in the Department of Oncology, Peking Union Medical College Hospital (PUMCH). A total of 49 healthy controls were enrolled in the study during the same period. The recruited patients were diagnosed with esophageal squamous cell carcinoma, gastric cancer, or colorectal adenocarcinoma by histopathology or cytology. All of the patients were confirmed as locally advanced or stage IV (AJCC 7.0) at the time of admission. Patients with previous treatment for cancer within 6 months before admission were excluded. The healthy participants in the control group reported no recent history of probiotic or antibiotic usage within 2 weeks. All of the subjects provided written informed consent prior to enrollment in this study. This study was approved by the ethics committee of Peking Union Medical College Hospital.

The participants' clinical data were collected by reviews of their medical records. The usage of antibiotics and probiotics was recorded in questionnaires. The whole blood cell test, lymphocyte subgroups, erythrocyte sedimentation rate (ESR), hypersensitive C-reactive protein (hsCRP), and antinuclear antibody (ANA) were tested in the tumor patients before treatment. Fecal samples of the patients were self-sampled one week prior to the start of anti-tumor therapy in the hospital. Fecal samples of the healthy controls were obtained at the Physical Examination Center of PUMCH. Then 50 to 100 mg fecal samples were placed in a MGIEasy sample collector. The sample bottle was immediately transferred to -80 °C for storage until testing.

DNA extraction and 16S rRNA gene sequencing

A NucleoSpin Soil DNA Kit (Macherey-Nagel, Germany) was used to extract fecal genomic DNA. The purified DNA was collected as template for PCR amplification using the primers of the V4 region^[16] of the 16S rRNA gene (515F: GTGCCAGCMGCCGCGGTAA^{806R}: GGACTACHVGGGTWTCTAAT). The purified PCR products were used for library pool construction and prepared for sequencing.

The qualified libraries were sequenced by a HiSeq2500 gene sequencing analysis system and PE250 sequencing strategy. The sequencing data of each fecal sample were 50000 sequence tags. The original sequencing data were collected for analysis.

Sequencing data analysis

The original sequencing data were filtered to remove low-quality sequencing fragments (reads). High-quality clean data were retained. The pairwise reads were merged into sequences (tags) by overlapping the relationships using Flash software (v1.2.11)^[17] (<http://ccb.jhu.edu/software/FLASH/index.shtml/FLASH-1.2.11.tar.gz>). USEARCH software (v7.0.1090) (<http://www.drive5.com/usearch>) was used to cluster the tags into the operational taxonomic units (OTU) with 97% similarity. The OTUs were assigned taxonomically to the Greengenes database (V201305)^[18] (<http://greengenes.secondgenome.com>) as references for species identification.

We estimated the alpha diversity using Shannon's index^[19] to evaluate the species diversity of each sample. Box-plots and rarefaction curves were drawn to show sequencing depth and diversity difference. Beta diversity was assessed by unweighted and weighted UniFrac distance^[20] to determine the dissimilarities of the two samples. Principal coordinate analysis (PCoA) and heat maps were used to visualize sample clustering by microbial community composition. Sample clustering in the diversity analysis was tested by an analysis of similarity (ANOSIM). The relative abundance of taxa at different bacteria levels was calculated and visualized using bar plots and heat maps. We conducted a linear discriminant analysis (LDA) effect size (LEfSe)^[21] (https://download.csdn.net/download/weixin_43585681/11530367) to analyze the differences in the relative abundance of taxa between the groups.

Functional metagenome prediction

We clustered the reads into OTUs using the Greengenes database as a reference. Reconstruction of the OTUs was conducted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)^[22] (<https://github.com/picrust/picrust>). Predicted functional genes were compared using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups of proteins (COG) databases to categorize and determine the abundance of metabolic pathways across the different groups.

Statistical analysis

R programming language (v 3.1.1) (<https://www.r-project.org>) was used for the statistical analysis. The Wilcoxon rank-sum test was used for the microbiota differences between two groups. The Kruskal-Wallis test was used to compare the microbiota differences among three or more groups. Quantitative data were expressed as mean±standard deviation and analyzed via SPSS 22.0 software (<http://www.onlinedown.net/soft/577760.htm?t=1492483499557>). The differences in the clinical data between the groups were compared using the t-test and analysis of variance (ANOVA). To analyze the bacteria differences between the sample groups, the false discovery rate (FDR) control was used for the multiple hypothesis test. $P < 0.05$ and $FDR < 0.05$ were considered statistically significant.

Results

Characteristics of the study subjects

From April 2018 to April 2019, 81 patients with GI tumors were enrolled (Table 1). The median follow-up time was 6 months. The median age was 63 years (29-75 years). A total of 24 cases with esophageal squamous cell carcinoma, 33 cases with gastric adenocarcinoma, and 24 cases with colorectal adenocarcinoma were included. Among the patients, 30 cases were identified as locally advanced tumors and 51 patients presented distant metastases. Nine patients were treated with antibiotics within 2 weeks before admission. A total of 33 patients had taken probiotics before admission. Overall, 49 healthy people were enrolled in the healthy control group with a median age of 55 years (35-70) and a male/female ratio of 1.33.

The baseline inflammatory markers ESR and hsCRP increased in 37 cases, and 14 cases were positive for ANA. The lymphocyte count in the peripheral blood decreased in 23 cases. Of all of the patients, 73 cases had baseline lymphocyte subgroup data. Some had significantly decreased T, B, natural killer cell (NK), or lymphocyte subgroups (Table 2).

Overview and quality control of 16S rRNA gene sequencing

In the present study, 130 fecal samples were analyzed. After sequencing and quality filtering, 11165611 tags were obtained. After clustering, the representative OTU sequences were obtained, and 1357 OTUs were generated. A total of 14 phylum levels of bacteria were detected in all of the samples: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia, Fusobacteria, Spirochetes, Synergistetes,

Chrysiogenetes, Cyanobacteria, Lentisphaerae, Tenericutes, TM7, and Elusimicrobia. The bacteria were further identified as 25 classes, 48 orders, 84 families, and 171 genera. A total of 132 species were identified. Nine patients treated with antibiotics before admission were excluded and analyzed separately.

To ensure the sequencing depth and sequencing quality, rarefaction curves were conducted for the alpha diversity (Supplemental Figure 1).

The fecal microbiota profile differed significantly between the GI cancers and controls

We analyzed the fecal microbiota of 72 patients without antibiotics usage and the fecal microbiota of the 49 healthy people in the control group. There were 23 cases of esophageal cancer, 28 cases of gastric cancer, and 21 cases of colorectal cancer included.

To evaluate the changes in the fecal bacteria between the patients with GI cancers and the healthy controls, we measured the alpha diversity within the samples and beta diversity between the samples. Shannon box plots (Supplemental Figure 2) indicated that the alpha diversity within each sample in both groups were balanced. We created PCoA plots on unweighted and weighted UniFrac distance matrices to evaluate the beta diversity. The bacterial composition of the GI cancer patients was somewhat different from that of the healthy group (ANOSIM $R=0.014$, $P=0.233$; $R=0.005$, $P=0.342$) for the unweighted and weighted distances (Fig 1A and B).

Comparing the OTUs with the Greengenes database, we created bacterial abundance bar plots and bacterial abundance heat maps at different classification levels of bacteria (phylum, class, order, family, genus, and species). Bacteroidetes, Firmicutes, and Proteobacteria accounted for 90% of the sequences. A comparison between the GI cancer and healthy groups found a higher relative abundance of Bacteroidetes and Proteobacteria and a lower relative abundance of Firmicutes and Actinobacteria in the GI cancer patients (Fig 2A). The bar plots (Fig 2B, C, and D) of esophageal cancer, gastric cancer, and colorectal cancer compared with the control group respectively showed similar bacterial differences at the phylum level. The average relative abundance analysis of the heat maps at the genus level demonstrated that the GI cancer patients had a higher abundance of *Bacteroides* (within Bacteroidetes), *Prevotella* (within Bacteroidetes), *Escherichia* (within Proteobacteria), and *Akkermansia* (within Verrucomicrobia) than in the control group. On the contrary, the abundance of *Bifidobacterium* within the Actinobacteria phylum and *Faecalibacterium*, *Ruminococcus*, *Clostridium*, *Blautia*, and *Roseburia* within the Firmicutes phylum were lower than those in the control group (Fig 3).

Overall, these results demonstrated that in the different taxonomic levels, the fecal microbial communities differed in the GI cancer patients and healthy controls. The major features of the fecal microbiota in esophageal cancer and gastric cancer are similar to those in colorectal cancer.

Specific microbial taxa were associated with GI cancer

We conducted an LEfSe analysis to identify the relevant taxa responsible for the differences between the GI cancer patients and healthy controls. We calculated the linear discriminant analysis (LDA) taxa score

to evaluate the effect of the taxa.

When comparing the genera differentially abundant in the GI cancer (n=72) and control groups (n=40) based on the LEfSe cladogram (Fig 4), an enrichment in *Bacteroides*, *Prevotella*, *Escherichia*, and *Akkermansia* were observed in the GI cancer patients. *Bifidobacterium*, *Faecalibacterium*, *Ruminococcus*, *Clostridium* and *Blautia* taxa were significantly more abundant in the control group. The results of the LDA score (Supplemental Figure 3) confirmed the genera differences between the groups.

We further identified the relevant species by conducting the Wilcoxon rank-sum test between the control group and different cancer groups (Table 3, Supplemental Figure 4). As shown in Table 3, *F. prausnitzii*, *B. adolescent*, and *C. clostridioforme* were significantly more abundant, around 2.3-9 times higher in control group (n=49) than in esophageal cancer group (n=23) with statistical significance ($P<0.05$, FDR<0.05). *B. fragilis* and *L. reuteri* significantly enriched in esophageal cancer were more prevalent in patients with esophageal cancer than in the healthy controls ($P<0.05$, FDR<0.05). We found significant decreases in the abundance of *F. prausnitzii*, *B. adolescent*, *C. clostridioforme*, *B. obeum*, *R. faecis*, and *D. formicigenerans* in the gastric cancer patients (n=28) compared with the healthy controls ($P<0.05$, FDR<0.05). The significant increases in the abundance of *P. corpi*, *E. coli*, and *L. reuteri* in the gastric cancer cases compared with the healthy controls were confirmed ($P<0.05$, FDR<0.05). In the colorectal cancer cases, we identified an enrichment in *B. fragilis* that was 2.1 times higher in abundance than healthy controls ($P<0.05$, FDR<0.05). *F. prausnitzii*, *B. adolescent*, *C. clostridioforme*, *R. gnavus*, *B. obeum*, and *R. faecis* were significantly more abundant in the control group than in the colorectal cancer group (n=21) with statistical significance ($P<0.05$, FDR<0.05). There was no significant difference in the fecal microbiota among the esophageal cancer, gastric cancer, and colorectal cancer groups.

Functional profile differed significantly between the GI cancer and controls

Based on the microbial community profiles obtained from the 16S rRNA gene sequences, we compared the functional genes in the KEGG and COG databases to infer the metagenomic functional changes. We applied the Wilcoxon test (Fig 5) and Kruskal test (Supplemental Table 1) to evaluate the functional differences between the GI cancer and control groups.

Regarding the physiological and biochemical metabolism, the predicted functional pathways significantly enriched in the GI cancer cases included lipid metabolism, coenzyme metabolism, inorganic ion metabolism, secondary metabolite catabolism, and glycan biosynthesis and metabolism ($P<0.05$, FDR<0.05). The enrichment of the functional pathways decreased in the GI cancer group in nucleotide transport and metabolism compared with the healthy control group ($P<0.05$, FDR<0.05). Regarding the microbial functional features involved in cell biological function, the results showed that the functional composition of the GI cancer microbiota had increased intracellular trafficking, secretion, vesicular transport, signaling interaction, RNA processing and modification, post-translational modification, protein turnover, and chaperone function ($P<0.05$, FDR<0.05) compared with the control group. Conversely, the enrichment in the function of membrane transport and cell cycle regulation decreased significantly ($P<0.05$, FDR<0.05) in the GI cancer cases. Regarding the metabolite differences at KEGG level 3

(Supplemental Table 1), the functional profile of the GI cancer bacteria showed significant enhancement in glycine, serine, threonine, and tryptophan metabolism, increased degradation of valine, leucine, and lysine, and increased functional abundance in inositol phosphate, arachidonic acid, glutathione, bacterial toxin, oxidative phosphorylation, phosphatidylinositol signal system, p53 signal pathway, and tricarboxylic acid cycle compared with the healthy control group ($P<0.05$, $FDR<0.05$). The functional abundance of lysine, valine, leucine, flavonoids, flavonol, and G protein coupled receptor in the tumor group significantly decreased compared to the control group ($P<0.05$, $FDR<0.05$). There was no significant difference in the synthesis of secondary bile acid and the function of the butyrate metabolic pathway between the two groups.

The correlation between peripheral blood markers and fecal microbiota differences

Among the 72 cases of GI cancer, 31 patients had elevated inflammatory markers ESR and hsCRP. The fecal microbiota in the patients with elevated markers (n=31) were more abundant with *P. tannerae* and *S. luteciae* compared to the patients with normal markers (n=41) ($P<0.05$, $FDR>0.05$). The abundance of *R. bromii* in the elevated inflammatory markers cases was lower than in the normal markers cases ($P<0.05$, $FDR>0.05$) (Supplemental Figure 5A). In the esophageal cancer patients, the abundance of *Veillonella dispar* in the cases with elevated inflammatory markers (n=11) was higher than in the normal markers (n=12), and the abundance of *B. uniformis* and *C. clostridioforme* was lower in the elevated marker cases (Supplemental Figure 5B). Compared with the fecal microbiota in the gastric cancer cases with normal markers (n=19), the gastric cancer cases with elevated markers (n=9) had a higher abundance of *B. eggerthii* and *Parabacteroides distasonis* and a lower abundance of *C. celatum* and *Rothia mucilaginosa* ($P<0.05$, $FDR>0.05$) (Supplemental Figure 5C). The fecal abundance of *Prevotella* and *V. dispar* in the patients with higher inflammatory markers (n=11) was higher than in the normal cases (n=10) ($P<0.05$, $FDR>0.05$) in the colorectal cancer group, while the abundance of *F. prausnitzii* was lower ($P<0.05$, $FDR>0.05$) (Supplemental Figure 5D).

ANA was positive in 12 GI cancer patients. Compared with the ANA negative patients (n=60), the ANA positive with high-titer ($\geq 1:160$) patients (n=3) had a significantly higher abundance of *Paraeggerthella hongkongensis* ($P<0.05$, $FDR<0.05$). The abundance of the taxa also tended to increase in the ANA low-titer positive group ($<1:160$) ($P<0.05$, $FDR>0.05$). The abundance of *R. lactaris* in the ANA positive cases was higher than in the ANA negative cases, while the abundance of *R. faecis* was lower in the ANA positive cases ($P<0.05$, $FDR>0.05$) (Supplemental Figure 6A and B).

The percentage of peripheral blood lymphocytes decreased at baseline in 19 patients (Table 4). The abundance of *S. luteciae* in the feces of the patients with lymphocytopenia was higher than in the patients with normal lymphocytes ($P<0.05$, $FDR>0.05$), while the abundance of *C. ramosun* in the patients with lymphocytopenia was lower ($P<0.05$, $FDR>0.05$). A total of 64 patients had lymphocyte subgroup data. The results showed that the abundance of *B. fragilis*, *C. sordellii*, and *C. spiroforme* in the feces of the patients with decreased CD4⁺ T cells (n=33) and naive CD4⁺ T cells (n=49) tended to be significantly lower than those in the normal group ($P<0.05$, $FDR>0.05$). The abundance of *Blautia* in the decreased

CD4⁺ T cell group was also lower than in the normal group ($P<0.05$, $P<0.05$). There were no significant differences between the patients with decreased and normal/increased CD8⁺ T cells ($n=32$), but the abundance of *B. adolescent* and *Eubacterium bifforme* in the patients with decreased CD8⁺CD28⁺ T/CD8⁺ T was lower than in the normal group ($P<0.05$, $FDR>0.05$). The abundance of *L. iners* in the patients with decreased numbers of B lymphocytes ($n=46$) tended to be lower in the patients with normal B cells ($P<0.05$, $FDR<0.05$). The abundance of *C. spiroforme* in the NK cell reduction group ($n=17$) was lower than in the NK increased group ($P<0.05$, $P<0.05$). (Supplemental Figure 7).

The influence of antibiotics, probiotics, and staging in the microbiota composition of GI cancer

A total of 28 patients had ingested probiotics within 2 weeks before enrolling in the study. Compared with the fecal microbiota of the other 44 patients, there were no significant differences in the abundance of dominant taxa between the two groups. Nine patients were treated with broad-spectrum antibiotics within 2 weeks before admission. Compared with the 72 patients without antibiotics, the results (Supplemental Figure 8) showed that the abundance of *C. clostridioforme*, *Dorea*, *Blautia*, and *R. bromii* tended to decrease in the antibiotic group ($P<0.05$, $FDR>0.05$). The abundance of *Roseburia* also decreased to 50% in the non-antibiotic group; however, there was no statistical difference ($P>0.05$). The abundance of *B. plebeius* in the antibiotic group was 2.63 times higher than in the non-antibiotic group, but the difference was not statistically significant ($P=0.06$).

To analyze the correlation between the fecal microbial profiles and GI cancer progression features, the feces of the GI cancer patients with locally advanced tumor ($n=29$) and distant metastasis ($n=43$) were assessed. The abundance of *C. clostridioforme* in the locally advanced staging group was 1.9 times higher than in the metastasis group ($P<0.05$, $FDR>0.05$), and the *C. colinum* abundance in the same genus was 10 times higher than in the metastasis group ($P<0.05$, $FDR>0.05$). Conversely, two species of the *Prevotella* genus (*P. tanneriae* and *P. melaninogenica*) in the metastasis group were higher than in the locally advanced staging group ($P<0.05$, $FDR>0.05$). The dominant taxa *B. plebeius* tended to decrease in the metastasis group (1.32% vs 4%, $P>0.05$) (Supplemental Figure 9).

Discussion

The microbial environment in the human gut is complex, and bacteria are the main components and the most studied. At present, approximately 90% of the normal gut microbiota in the human body consist of Firmicutes and Bacteroidetes at the phylum level, while the other 10% are mainly composed of Actinobacteria, Proteobacteria, and Fusobacteria^[23]. Some studies have demonstrated that there may be significant differences in gastrointestinal microbiota between cancer patients and healthy people. In this study, 16S rRNA sequencing was used to analyze the characteristics of fecal microbiota in patients with different types of GI tumors and identify the correlation between microbiota and clinical factors.

We identified significant differences in the fecal microbial communities in 72 cases of GI cancer and the healthy control group. The microbiota profile in the cancer group showed a significant decline in the

abundance of Firmicutes and Actinobacteria, while the abundance of Bacteroidetes, Proteobacteria, and Verrucomicrobia was significantly higher than in the control group. Previous studies found that Bacteroidetes increased (16.2% vs 9.9%) and Firmicutes decreased (74.0% vs 80.3%) in the gut of patients with colorectal cancer compared with healthy people^[24], which was partly consistent with the results of the present study.

At the species level, we demonstrated that the abundance of *F. prausnitzii*, *C. clostridioforme*, and *B. adolescent* in the different cancer groups was significantly lower than in the healthy controls. Previous research reported that the dominant microbiota *F. prausnitzii* can produce butyrate. Butyrate inhibits colonic inflammation and prevents carcinogenesis by blocking the nuclear factor kappa beta (NFκB) signaling pathway and inducing the activation of T cells. This study also demonstrated the reduction in the abundance of *F. prausnitzii* in the feces of the colorectal cancer patients compared with the healthy subjects with statistical significance^[25]. *C. clostridioforme* activates the intracellular signaling pathways in the early stage of inflammatory response^[26], and has been found to reduce in the feces of patients with colorectal cancer^[27]. Melanoma-bearing mice with a high abundance of *Bifidobacterium* in the gut demonstrated better response to immunotherapy so that *Bifidobacterium* was considered a protective bacteria^[13]. In addition to the three bacterial taxa that decreased in the different kinds of GI cancer groups compared with the control group, the abundance of *Ruminococcus gnavus*, *Roseburia faecis*, and *Blautia obeum* was significantly lower in the gastric cancer and colorectal cancer groups. The abundance of *Dorea formicigenerans* in the gastric cancer group decreased in comparison with the healthy controls with statistical significance. Partly supporting our data, various studies reported the reduction in *Ruminococcus* and *Roseburia* genera in the feces of colorectal cancer patients compared with healthy people^[28,29]. *Roseburia* can ferment dietary fiber into butyrate, which was confirmed to be a vital protector of the gut^[8]. The abundance of *Blautia* in the intestinal mucosa of colorectal patients was reported to be lower than in healthy people^[30], but the data on this taxa in feces are deficient. A study of 18 patients with rectal cancer compared with 18 healthy people^[31] showed that *Dorea* in the rectal tissue was more abundant in rectal cancer patients, which was the opposite of the results of the present study. The reason for this inconsistency may be related to the different sources of samples. At present, there is a lack of research on gut microbiota in patients with esophageal and gastric cancer. It has been reported that dysbiosis in esophageal and gastric tissues is related to tumorigenesis. The results of an animal model study showed that the abundance of *Bifidobacterium* in the stomach decreased significantly after HP infection^[32]. In summary, the present study found for the first time that the profile of the gut bacteria in patients with upper digestive tract tumors, mainly characterized by the consumption of butyrate-producing bacteria such as Firmicutes, were basically consistent with previous research on colorectal cancer microbiota. This suggests that these beneficial bacterial taxa, which have antagonistic effects on the pathogenesis of lower digestive tract tumors, may also be closely involved in the occurrence of esophageal and gastric cancer.

Among *B. fragilis*, *Prevotella*, *E. coli*, *L. lactis*, and *A. muciniphila*, all were dominant bacteria in the gut except *Lactobacillus lactis*. Their abundance in the cancer groups was significantly higher than in the

control group. Contrary to the decrease in butyrate-producing bacteria, the abundance of *Bacteroides* in the feces of patients with colorectal cancer and intestinal adenoma increased in previous reports^[33]. The enterotoxin secreted by *B. fragilis* was significantly higher in the intestinal mucosa and feces of patients with colorectal cancer than in healthy people, and positively correlated with disease progression^[34]. It was suggested that this enterotoxin stimulated E-cadherin, beta-catenin, NFκB, and signal transducer and activator of transcription 3 (STAT3) to promote the proliferation of intestinal epithelial cells and the expression of c-myc oncogene, which leads to carcinogenesis of the large intestine^[35]. *E. coli*, *Prevotella*, and *A. muciniphila* were also found to be increased in the feces of patients with colorectal cancer^[36, 37]. *Prevotella* were detected in the cancer tissues of patients with esophageal cancer, and the higher the abundance, the worse the prognosis^[38]. HP and *E. coli* in cancer tissues were associated with the occurrence of esophageal carcinoma^[39]. The abundance of *E. coli* in the stomach of patients with gastric cancer was also higher than in healthy people^[40]. The basic abundance of *Lactobacillus* in the human gut is relatively low. The reason for the overabundant result of *Lactobacillus* in the gastric cancer group may due to the high percentage of cases with probiotics usage before admission (46.4% in the gastric group vs 30% in the esophageal and colorectal cancer groups). Based on our data and earlier reports, we found that the increased fecal bacterial taxa in patients with esophageal and gastric cancer were similar to those in patients with colorectal cancer.

By prediction of the functional genes, the PICRUST analysis inferred the functional capacity of the microbiota. We found that the gut microbiota of the patients with GI cancer and healthy people had significant differences in the function of different metabolic pathways. The enhanced function of the phosphatidylinositol signaling pathway in the tumor group was involved in the regulation of tumor necrosis factor-α (TNF-α). TNF-α can induce the expression of cyclooxygenase-2 (COX-2). Enterotoxins secreted by overabundant *B. fragilis* in the tumor group can also activate NFκB to upregulate the synthesis of COX-2, which has been shown to be closely related to the transformation from colonic inflammation into cancer^[35,41]. Arachidonic acid is formed by the synthesis of dietary linoleic acid in food. Its metabolites have pro-inflammatory properties. It has been proved to increase in the intestinal mucosa of patients with ulcerative colitis^[42]. We demonstrated that the metabolic function of arachidonic acid in the cancer group was stronger than in the control group. Whether this metabolic process is directly related to GI cancer needs further study. Flavonoids have been proved in various studies to inhibit the proliferation and migration of cancer cells, inhibit angiogenesis, inhibit the growth of *E. coli*, promote the growth of beneficial bacteria, and regulate the balance of intestinal microbiota, thus having a preventive effect on various tumors^[43]. We found that the biosynthesis of flavonoids in the GI cancer group was significantly lower than in the healthy controls. We inferred that the microbial characteristics in patients with GI tumors may interfere with the synthesis of flavonoids. Gut microbiota can convert tryptophan into indole and other metabolites. These metabolite products can regulate the intestinal mucosal barrier function and immune system. It is reported that the increase in tryptophan in the blood can be used as a potential biomarker for colorectal cancer^[44]. In the present study, the enhanced metabolic function of tryptophan in the cancer group indirectly supported this hypothesis, indicating that tryptophan and its

metabolites may be related to the pathogenesis of GI tumors. Research on the correlation between short-chain fatty acids (SCFA) such as acetic acid, butyric acid, and microbiota has recently increased. It is thought that SCFA can bind to G protein-coupled receptors in the intestine, regulate immunity, alleviate inflammation, and prevent tumorigenesis^[25]. The results of the present study supported previous reports, as the abundance of butyrate-producing bacteria and the functional abundance of G protein-coupled receptors in the healthy group were significantly higher than those in the cancer group. However, the metabolic function of butyrate did not show statistical difference between the two groups. We speculated that the metabolism of butyrate may be affected by many factors. The GI cancer groups showed hyperfunction in cellular signal transduction, which may contribute to the biological behavior of cancer. At present, the study of gut microecology and metabonomics has become a popular topic. The mechanism of microbiota involvement in metabolism and cellular biological functions is still unclear in the field of digestive tract cancer. It may become a trend in future research to explore the biological behavior and even prognosis of patients with GI cancer through the functional analysis of key microbiota.

Human gut microbiota is affected by many factors. Although we have explored the bacterial differences among different levels of peripheral blood markers and disease staging of patients, due to the existence of confounding factors, the clinical significance of the differences still needs further verification. The feces of the cancer patients with later staging had a higher abundance of *Prevotella* and fewer *Clostridium*, similar to the differences between cancer patients and healthy people. These beneficial or harmful taxa may also be related to the blood markers of patients with GI tumors.

Tumor patients have been considered to have immune tolerance and abnormal lymphocyte function. Studies have shown that the number and function of lymphocytes are related to the prognosis of tumor patients. Some gut microbiota can regulate the function of T lymphocytes and NK cells^[45]. For example, the protein produced by *C. nucleatum* can bind to the receptors of T and NK cells and block the cytotoxicity of immune cells to attack intestinal cancer cells^[46]. The antigen of *Clostridium* can be recognized by FOXP3⁺CD4⁺ T regulated T cells (Treg) and become intracolonic Treg, which helps inhibit inflammation^[47]. Our study found that the abundance of several *Clostridium* species in the feces of the patients with peripheral blood CD4⁺ T, naïve CD4⁺ T, and NK cell reduction tended to decrease, which may be due to the regulatory effect of *Clostridium* on colonic Treg, but there have been few reports about the effects of *Clostridium* on blood lymphocytes. Some researchers have investigated the response of CD4⁺ T cells in the blood and gut to gut bacteria in patients with inflammatory bowel disease. The results showed that the number of CD4⁺ T cells in the blood and gut both increased, but there was functional heterogeneity^[48]. The number of peripheral blood lymphocytes is not only affected by the cancer progression and microbial exposure, but also by age-related alterations. Further investigation of cytokines and lymphocyte function may be a promising and useful supplement to this study.

This study had some limitations, such as the limited sample size, short follow-up time to obtain only short-term efficacy data, and no follow-up to survival time. The common problem in the study of microbiota in the human population is that fecal bacteria are affected by many environmental factors,

which may bias the results. In future research, we should increase the sample size, improve the research design according to the results of this study, prolong the follow-up time, and establish animal models combined with more in-depth metabolomics and immune cell function analysis to further evaluate the characteristics and functions of gut microbiota in patients with GI tumors and explore the microbial biomarkers for prognostic value.

Conclusion

Esophageal cancer and gastric cancer patients have similar fecal microbial features with colorectal cancer patients, which are mainly characterized by the significant consumption of butyrate-producing bacteria and the increase in some specific bacteria taxa. The 16S rRNA microbial analysis method in fecal samples is of great clinical value for evaluating the intestinal microecological characteristics of patients with upper digestive tract tumors. Differential bacterial taxa in the feces of patients with GI cancer and healthy people may contribute to the pathogenesis of cancer through their metabolites and their effects on the cellular functional pathways. There are quantitative differences in peripheral blood lymphocytes and their subgroups in patients with GI tumors. Studies suggested that the differences in lymphocytes may be correlated with the composition characteristics of fecal microbiota. Our study provided insights into fecal microbiota composition and function profiles in GI tumor patients. The results encourage more research into the intricate host-microbiota relationship in patients with GI cancer.

Abbreviations

GI:gastrointestinal; rRNA: ribosomal RNA; PD-L1:anti-programmed cell death ligand-1; PD-1: anti-programmed cell death receptor-1; PUMCH: Peking Union Medical College Hospital; ESR: erythrocyte sedimentation rate; hsCRP: hypersensitive C-reactive protein; ANA: antinuclear antibody; OTU: operational taxonomic units; PCoA: principal coordinate analysis; ANOSIM: analysis of similarity; LDA: linear discriminant analysis; LEfSe: linear discriminant analysis effect size; PICRUST: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; KEGG: Kyoto Encyclopedia of Genes and Genomes; COG: Clusters of Orthologous Groups of proteins; ANOVA: analysis of variance; FDR: false discovery rate; NK: natural killer cell; NF- κ B: nuclear factor kappa beta; STAT3: signal transducer and activator of transcription 3; TNF- α : tumor necrosis factor- α ; COX-2: cyclooxygenase-2; SCFA: short-chain fatty acids; Treg: regulated T cells

Declarations

Ethical approval and consent participate

This study was approved by the Ethics Committee of Peking Union Medical College Hospital. All participants approved to participate, and written informed consent was obtained.

Consent for publication

All of the co-authors consented to publish this manuscript. A copy of the written consent is available for review.

Availability of data and material

Please contact author for data requests

Competing interests

The authors declare that they have no competing interests.

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Author contributions

NL designed the study, analyzed the data, and wrote the manuscript. CB, YZG, and LZ contributed to the data interpretation and the revision of the manuscript. NL, YPG, and XL performed the sample collection. All authors read and approved the final manuscript.

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Authors' information

Ningning Li: Department of Oncology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100730, China; rain.cmu@163.com;

Chunmei Bai: Department of Oncology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100730, China; baichunmei1964@163.com;

Yuanzhi Guan: Department of Microbiology & Parasitology, Peking Union Medical College, Beijing 100005, China; guan365@126.com;

Lin Zhao: Department of Oncology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100730, China; wz20010727@aliyun.com;

Yuping Ge: Department of Oncology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100730, China; geyp@pumch.cn;

Xiaoyuan Li: Department of Oncology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100730, China; lixiaoyuan1976@126.com.

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Figures

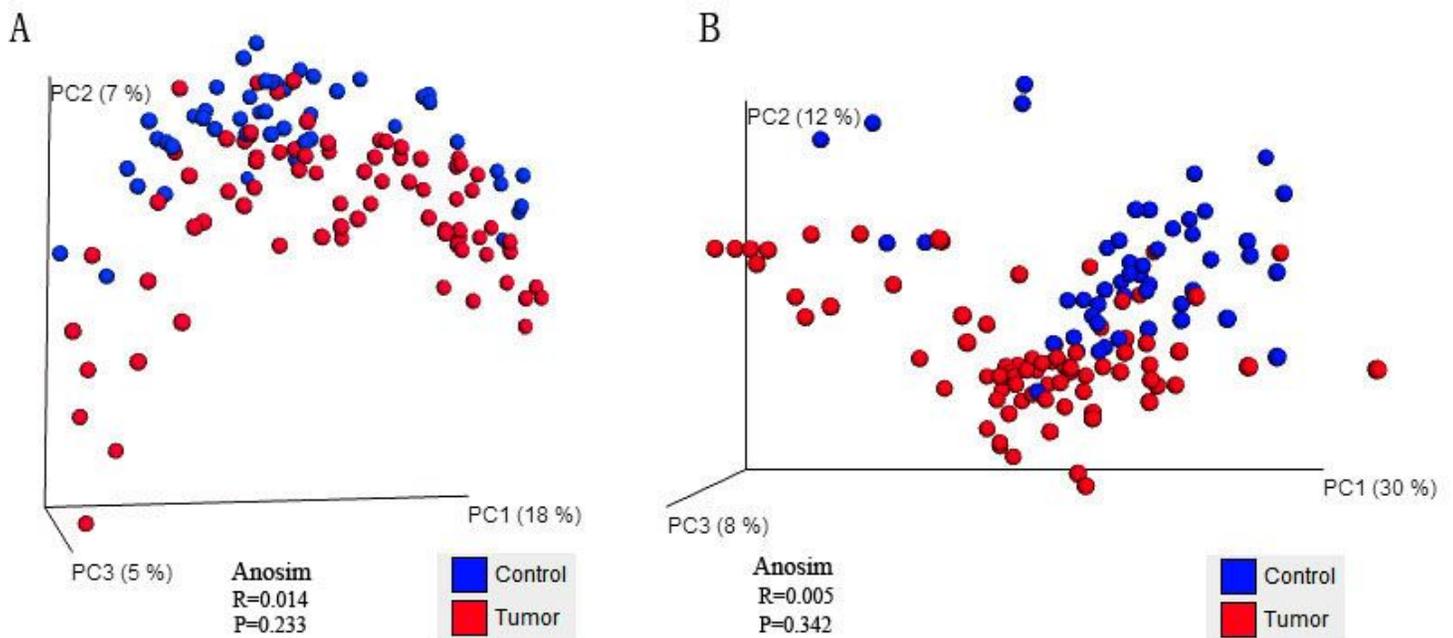


Figure 1

The fecal microbiota profile differs in GI cancer (Tumor) and healthy controls (Control). Principal coordinate analysis (PCoA) plots of (A) unweighted and (B) weighted UniFrac distances in which the samples are colored by different groups. The percentage of microbial diversity captured by each coordinate is shown. ANOSIM, analysis of similarity.

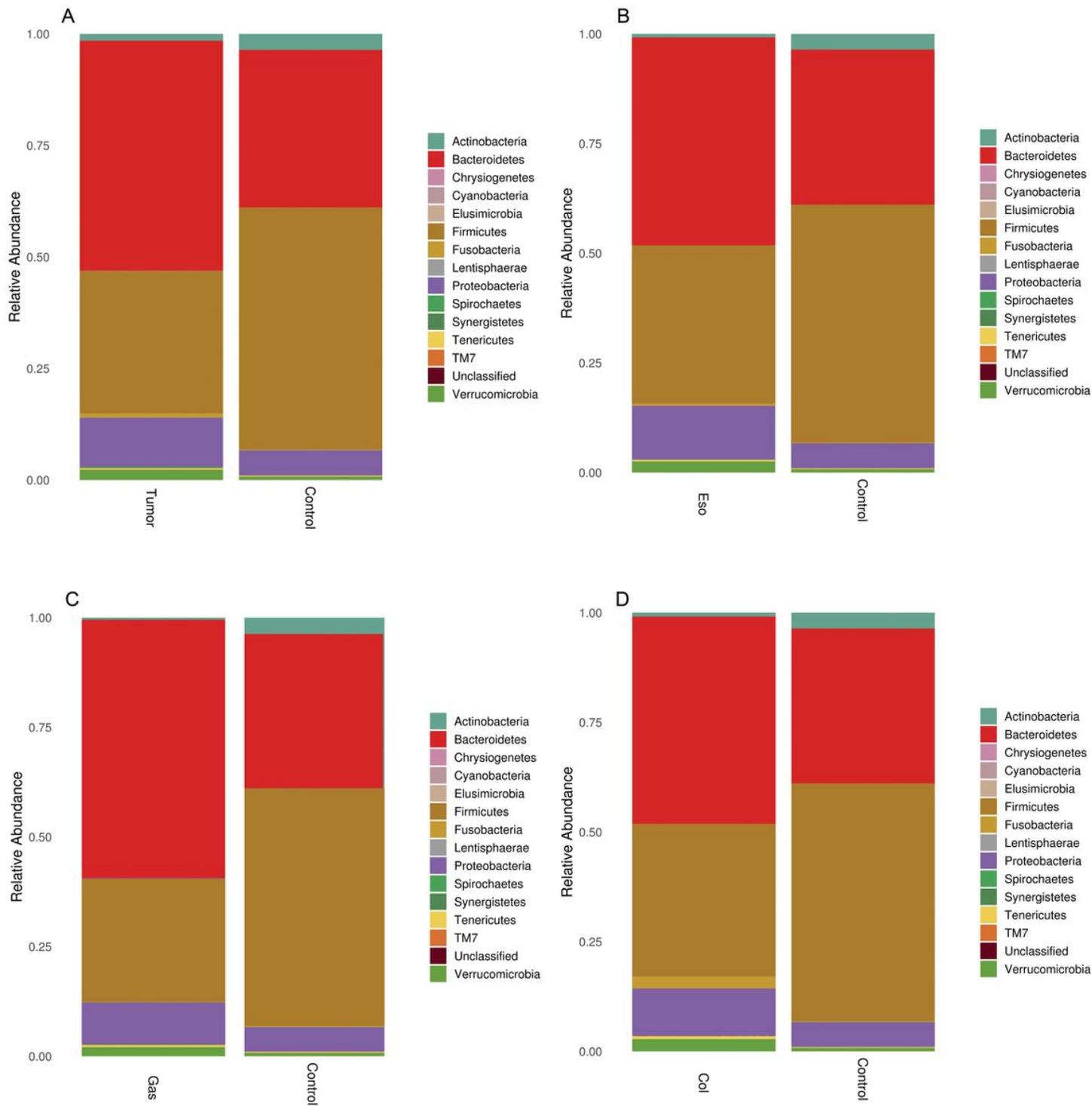


Figure 2

Relative abundance of the main bacterial phylum in the feces of (A) all GI cancer patients (Tumor) and healthy controls (Control), (B) esophageal cancer patients (Eso) and healthy controls (Control), (C) gastric cancer patients (Gas) and healthy controls (Control), and (D) colorectal cancer patients (Col) and healthy controls.

Relative Abundance

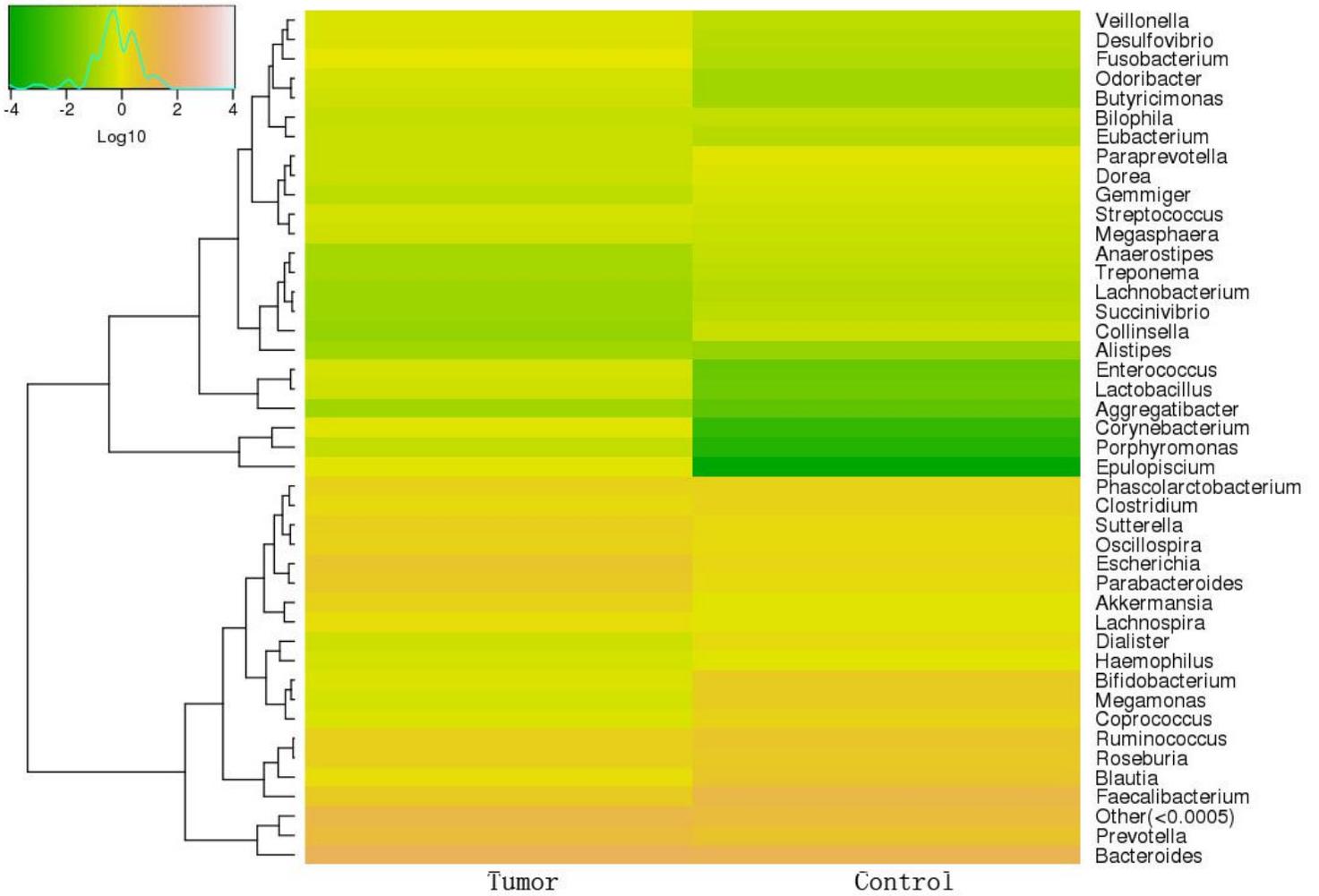


Figure 3

Mean genus-level OTU abundances are expressed as a heat map in GI cancer (Tumor) and healthy controls (Control).

Cladogram

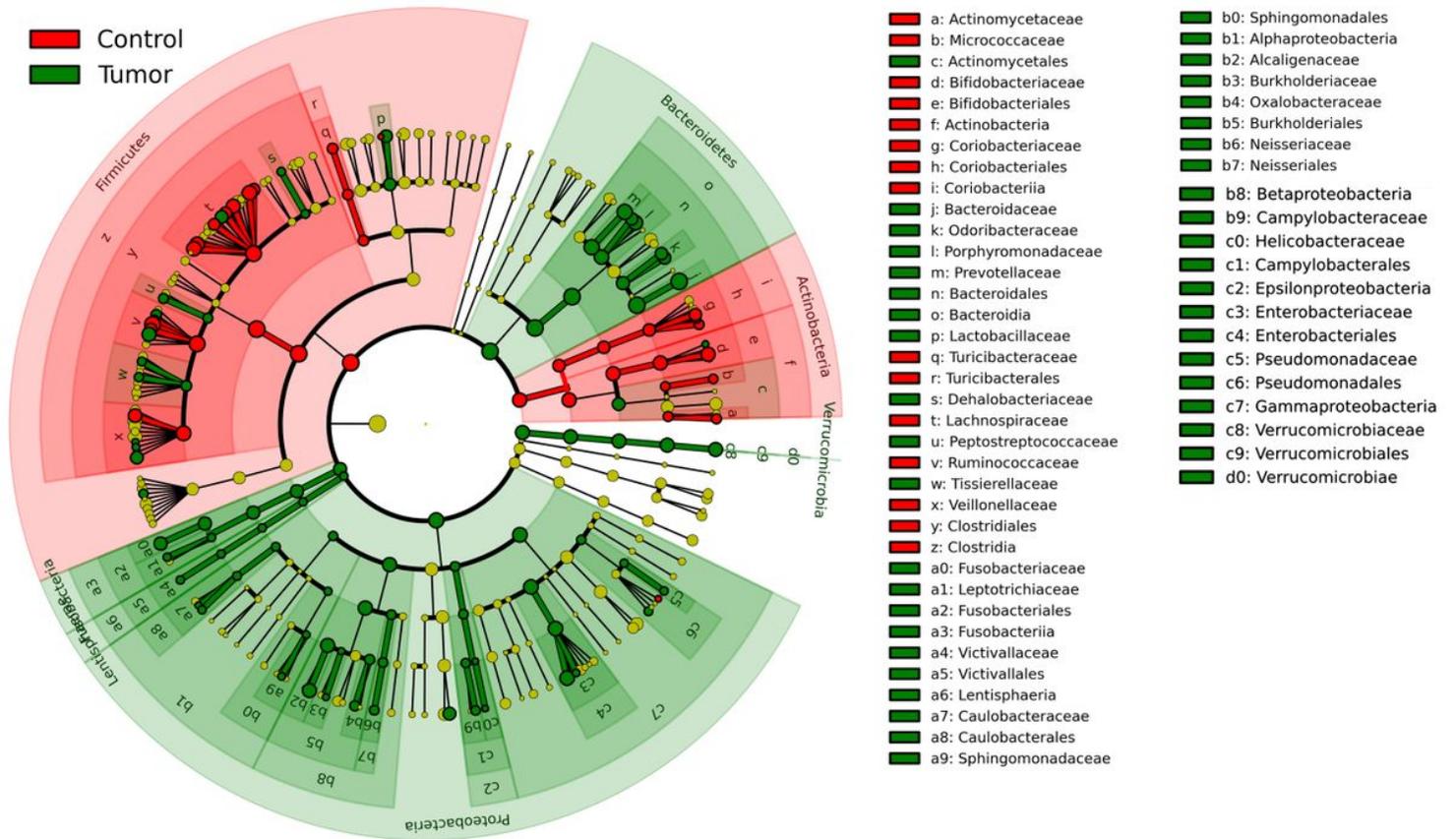


Figure 4

LEfSe cladogram representing the microbiota genera taxa associated with GI cancer group (Tumor) and healthy controls (Control).

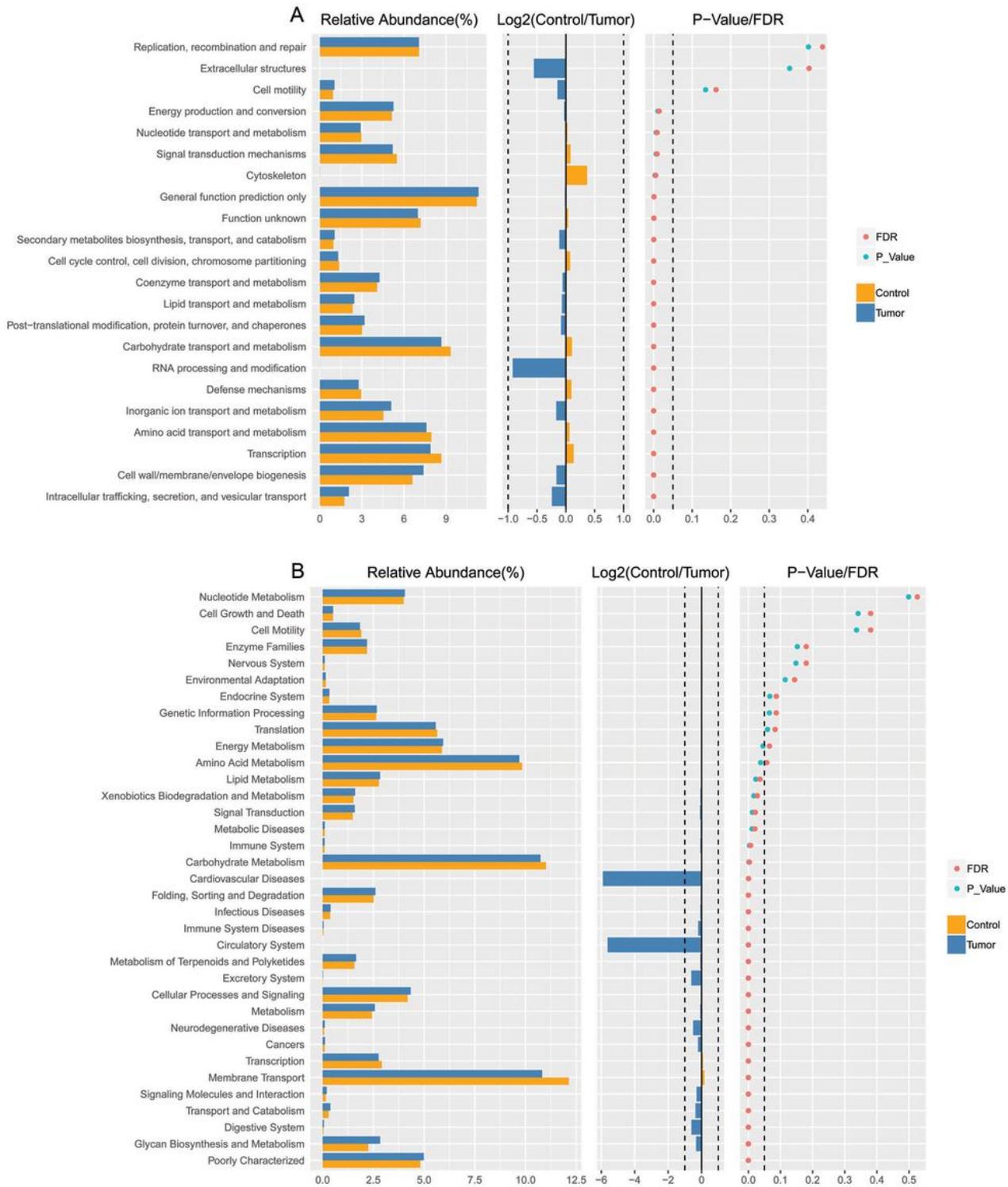


Figure 5

Functional profiles of the predicted metagenome of the bacterial communities of cancer cases (Tumor) and healthy controls (Control) evaluated by (A) KEGG pathway analysis and (B) COG pathway analysis. Significance was considered at $P < 0.05$ for the Wilcoxon test and FDR adjusted $P < 0.05$.

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