Gut Microbes and Microbial Metabolites in Colorectal Cancer Complicated with Different Serum Albumin Levels

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Abstract

Background

Patients with colorectal cancer (CRC) are at risk of malnutrition. Gut microbes and microbial metabolites are involved in the initiation and development of CRC.

Purpose

To investigate serum protein levels in CRC patients and explore the role of gut microbes and microbial metabolites in CRCs complicated with different serum albumin levels.

Methods

Overall, 398 CRC patients and same number of healthy volunteers in Huzhou Central Hospital from January 2016 to December 2018 were recruited to compare serum protein levels. The serological indicators were detected by Abbott Automatic Biochemical Analyzer(HCHL-YQ-SH-01). A total of 30 and 56 stool samples from CRC patients were used to detect intestinal microbes and microbial metabolites, respectively. Bacterial 16S V3-V4 and fungal ITS ribosomal DNA genes were sequenced and gas chromatography/mass spectrometry (GC/MS) was performed to detect microbial metabolites.

Results

Some serum protein-related indicators in the CRC group were lower than those in the control group (p□0.05). The total protein and albumin levels in colon cancer patients were lower than those in rectal cancer patients (p□0.05). The higher abundance of *Sutterella* is correlated with lower serum albumin level in CRCs. There were statistically significant differences in the abundance of fungi including *Agaricomycetes, Simplicillium, Sclerotiniaceae, and Exophiala* among patients with different serum albumin levels. Multiple gut bacteria and fungi are closely related to serum albumin levels.We found some characteristic microbial metabolites in CRCs complicated with different serum protein levels.

Conclusions

The different serum albumin levels were associated with the gut microbes and microbial metabolites in CRCs. It may provide novel ideas for basic research and clinical application.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and the incidence of CRC has increased in recent years[1]. With advances in multidisciplinary comprehensive treatment (MDT), molecular targeting treatment and immunotherapy, the 5-year survival time and 10-year survival time of CRC patients has reached over 30% and 20%, respectively[2–4]. CRC is a disease with insidious pathogenesis, with patients often experiencing a series of pathophysiological changes such as inflammation, ulcers, hemorrhage, and intestinal obstruction that inhibits the absorption of nutrients by the body. CRC cells require a large amount of nutritional support in the rapid proliferation phase resulting in the depletion of nutrients in the body. Therefore, CRC patients are at risk of malnutrition at an early stage of the disease. Malnutrition has adverse effects on the choice of surgical timing, postoperative recovery, immune function, incidence of complications, and hospitalization costs. Therefore, the early risk assessment of malnutrition is more beneficial than intervention after the occurrence of malnutrition.

Many studies supported that gut microbes[5–7] and microbial metabolites[8, 9] are involved in the occurrence and development of CRC. The main clinical symptoms of malnutrition include fatigue, emaciation, and edema, and it is caused by the consumption of carbohydrates, fats, and proteins. We have systematically summarized the association network between the gut microbes and metabolism of nutrients including carbohydrates, fat and protein in the form of literature review[10]. Previous studies have found that CRCs are often complicated with dyslipidosis and there were differences in gut bacteria among CRC patients with different lipid metabolism levels[11]. However, the correlation between intestinal microecological environment and protein levels in CRC patients remains to be confirmed.

Many studies have reported that high-protein diets leaded to changes in gut microbes and microbial metabolites. For instance, a dog model showed that high-protein diet increased the abundance and activity of butyrate-producing bacteria in stool samples[12]. Jianping, et al. reported that the high-protein diet increases the abundance of *Bacteroidetes, Prevotella, Oscillospira* and *Sutterella* bacterial species, and decreases the abundance of *Firmicutes*[13]. Chunlong, et al. reported that high-protein diet can decrease the numbers and activity of propionate- and butyrate-producing bacteria[14]. Xiangyu, et al. reported that high-protein diet could cause an increase in protein fermentation and lead to increased production of detrimental metabolites[15]. Carlo, *et al*. reported that high-protein diet can increase the concentration of ammonia in canine feces[16]. Floor, et al. reported that the gut microbes including *Lachnospiraceae*, *Erysipelotrichaceae*, and *Clostridiaceae* families are involved in protein catabolism[17]. Moreover, microbial metabolites including SCFAs increased in rats fed with high protein diets[17].

The nutrients such as protein, fat and carbohydrate can change the community structure and diversity of intestinal microorganisms[10]. The nutrient metabolism of nondigestible food components, such as SCFAs, polyamines, polyphenols, and vitamins, produced by intestinal microorganisms, can reprogram the genome through epigenomic mechanisms[18].To sum up, we propose a hypothesis that intestinal microorganisms and microbial metabolites may be related to host nutrient metabolism. The hypothesis may not only provide a new direction for early risk prediction of malnutrition, but also provide novel ideas for the study of nutrition supply in the process of CRC cell proliferation. Clinical investigation shows that

serum protein levels in CRC patients are lower than that in the healthy volunteers. To further confirm this hypothesis, we analyzed the gut microbes and microbial metabolites in CRC patients with different serum albumin levels in the present study.

Materials And Methods

1. **Study design**

The study design is shown in **Supplement material-figure S1**. There are 3 cohorts in this study. Cohort 1, 398 CRC patients at Huzhou Central Hospital from January 2016 to December 2018 were retrospectively analyzed, and 398 age-matched healthy subjects were included from the physical examination center, used to view the differences in serological characteristics between CRC and healthy individuals. Cohort 2, 30 stool samples from CRCs were taken to investigate the gut bacteria and fungus according to different serum albumin level. Cohort 3, 56 stool samples from CRCs for the microbial metabolites were investigated according to different serum albumin level.

2. **Subjects**

The patients admitted to the Huzhou Central Hospital and healthy volunteers from the physical examination center in Huzhou Central Hospital were recruited in the study as subjects. The included patients were all Han nationality, all living in Huzhou city, Zhejiang province,
People's Republic of China, with similar living habits and eating habits. The clinical trials involving the CRC patients and healthy volunteers and the informed consent form were approved by the Chinese Clinical Trial Registry (http://www.chictr.org.cn, No. ChiCTR1800018908) and Ethics Committee of Huzhou Central Hospital (No.201601022, No.20190204). The inclusion and exclusion criteria of subjects with regard to clinical investigation are shown in **Supplemental material 1.** All of the stool samples were taken from the above 398 CRC patients in cohort 1. More, patients with known complications such as diabetes, hypertension, smoking, and drinking history over the course of 1 year were excluded in the study of microorganisms and microbial metabolites. The samples used for microbial metabolites study (n=56) covered the samples used for gut microbiota sequencing (n=30). The final included samples for gut microbiota sequencing were those that excluded DNA extraction failure and those that did not meet quality control standards in gut microbiome study.

3. Clinical data acquisition and stool sample collection

Basic information and serological indicators with regard to the retrospective study were obtained from the medical record management system of Huzhou Central Hospital and health records system of the physical examination center. The serological indicators were detected by Abbott Automatic Biochemical Analyzer(HCHL-YQ-SH-01). The contents of serum albumin and total protein were determined by bromocresol green method and biuret method, respectively. The results of serological indexes were obtained from the clinical laboratory of Huzhou Central Hospital(Huzhou, Zhejiang province, China). The UICC/AJCC TNM staging system (Version 8, 2017) was used as a standard for

clinical staging[19]. Approximately 100 g stool sample was collected and stored in a -80 °C freezer within half an hour. The stool samples were analyzed within three months. Freezing the stool samples within half an hour and analyzing within three months was intended to reduce the experimental errors caused by the changes of microorganisms and their metabolites in vitro.

4. **Gut microbe detection**

4.1. DNA extraction and PCR amplification

Total DNA from the stool samples was extracted by using the E.Z.N.A.® Stool DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.). DNA integrity was verified using nanodrop ND-1000 spectrophotometer (LabTech, Washington, DC, USA) with the absorbances at 260 nm and 280 nm (A260/A280). The quality of the purified DNA was assessed by 2.0% (w/v) agarose gel electrophoresis. The V3–V4 region of the bacterial 16S ribosomal DNA gene and fungal ITS ribosomal DNA gene were amplified by PCR. The primers were as follows: bacterial 16S rDNA(630bp), F 5'-CCTACGGGNGGCWGCAG-3' and R 5'-GACTACHVGGGTATCTAATCC-3'; ITS rDNA(386bp): F 5'-CTTGGTCATTTAGAGGAAGTAA-3' and R 5'-GCTGCGTTCTTCATCGATGC-3'. The PCR reaction of 25 μl total volume comprised the DNA template (5 μl), Nextera XT Index Primer 1 (10 M, 2 μl), Nextera XT Index Primer 2 (10 M, 2 μl) and ddH_2O (16 μl). The conditions for PCR were as follows: 95℃ for 3 min, 25 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 45 s and 72℃ for 5 min.

4.2. MiSeq library construction and sequencing

The amplicons were extracted, purified and quantified by using 2% agarose gels, AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and QuantiFluor™-ST, respectively. The products were ligated with Y adapter and the self-ligated Y adapters were removed by using magnetic nanoparticles. PCR amplification was conducted for library enrichment and treated with NaOH solution. The single-stranded DNA denatured by NaOH were used to construct an Illumina Pair-End library. The Illumina MiSeq platform was used to sequence the amplicon library (Shanghai BIOZERON Co., Ltd.). The raw reads were deposited into the Read Archive (SRA) database of NCBI Sequence (Huzhou Central Hospital ： SUB4802613 SRP151510 :PRJNA478277; Huzhou Central Hospital ： SUB4648640 SRP169843 : PRJNA506089).

4.3. Bioinformatics analysis

The sequencing data were filtered and trimmed, then used for clustering OTUs (operational taxonomic units) and taxonomic analysis. The raw data were optimized as follows: sequences without primers were removed with an allowable error of 0.15 (cutadapt, version 1.11), then PE reads were assembled with an allowable overlap longer than 10 bp (pandaseq, version 2.9) and the ambiguous bases, sequences longer than 300–480 bp, or reads with average quality score less than 20 were discarded. The SILVA database[1] and the UNITE database were used to analyze and compare the clean reads of 16S rDNA gene sequences and ITS rDNA gene sequences, respectively. The Chao1 indexes, Shannon indexes, and Simpson indexes

were used as indicators for the bacterial alpha diversity analysis. Rarefaction curve is a plot of the number of OTUs as a function of the number of sequencing reads. The flattening of the rarefaction curve indicates that the sequencing depth is appropriate. The Shannon and Simpson index indicates the level of microbial diversity of the sample. Higher Shannon and Lower Simpson value indicates higher community diversity. The Mothur software was used to acquire the taxonomy information. RDP-classifier software annotates, Qiime software, and R package HCLUST (http://sekhon.berkeley.edu/stats/html/hclust.html) were used to annotate, cluster OTUs, and analyze diversities and community structures.

5. Microbial metabolites detection

5.1. Sample pretreatment

After screening and identification, 56 cases of CRC patients were enrolled to perform the gut microbial metabolite detection. Patients included in gut microbial metabolites detection were divided into three groups according to albumin levels. M0, M1, and M2 represent ranges of albumin values of over 40 g/L, 35~40 g/L and below 35 g/L, respectively. The samples were pretreated with metabolites extraction, desiccation and derivatization to analyze the microbial metabolites of the stool samples. Stool samples (10 mg) were mixed with NaOH solution (300 μl) and homogenized using a homogenizer (BB24, Next Advance, Inc., Averill Park, NY, USA). The supernatant (200 μl) was collected from the mixture after centrifuging at 4°C and 16000 rpm for 20 min (Microfuge 20R Beckman Coulter, Inc., Indianapolis, IN, USA). The residue was homogenized with cold methanol (200 μl), centrifuged and 167 μL of supernatant was collected. A robotic MultiPurpose Sampler MPS2 with dual heads (Gerstel, Muehlheim, Germany) was used to automatically derive the supernatants. The process was as follows: methyl chloroformate (20 μl) was added to the mixture that was shaken for 30 seconds; this step was repeated for the second derivatization; $CHCl_3$ (400 ml) and Na_2CO_3 solution (50 mmol*400 ml) were added, the mixture was centrifuged at 4°C, 4000 rpm for 20 min; the $CHCl_3$ layer at the bottom was transferred to an auto-sampler vial preloaded with anhydrous Na_2SO_4 (25 mg), shaken at 4°C, 1500 rpm for 20 min and transferred to a capped empty auto-sampler vial for injection.

5.2. GC/TOFMS analysis

Each 1 μl derivatized sample was injected into an Agilent 6890N gas chromatography coupled to time-of-flight mass spectrometry (GC-TOFMS) system (Pegasus HT, Leco Corp., St. Joseph, MO, USA). Separation was performed on Rxi-5MS capillary column (Crossbond ® 5% diphenyl/ 95% dimethyl polysiloxane) 30 m (length) x 250 μm I.D., 0.25 μm film thickness). Helium (99.9999%) with a flow rate of 1.0 mL/min was the carrier gas. The GC temperature gradient was the following: 45°C (1 min), 45-260°C (20°C/min), 260-320°C (40°C/min) and 320°C (2 min). The electron impact (EI) ion was held with a filament bias of -70 eV at a mass range of 38–550 Da and at 220°C. The acquisition rate was 20 spectra/second in the MS setting.

5.3. Differential metabolites identification

The sample quality control was referred to criterion (ISO9001, QAIC/CN/170149, Metabo-Profile, Shanghai, China). The XploreMET software (v2.0, Metabo-Profile, Shanghai, China) was used to process the raw data from GC/TOFMS analysis for automatic baseline correction, smoothing, peak picking, peak signal, library searching, and area calculation. The differential metabolites were identified as follows: The GC/MS workstation software was used to determine charge ratio and abundance of characteristic ion fragmentation patterns by comparing the fragment mass automatically. The fragment mass made reference to the available reference standards in the lab (Metabo-Profile Biotechnology Co. Ltd., Shanghai, China), the NISI II standard mass spectral databases, and the Fiehn databases linked to the LECO ChromaTOF software. A similarity of more than 70% was used as reference standard.

6.Statistical analysis

The variables were described with mean ± standard deviation (SD) or median value and analyzed using the Student's t-test or SNK test, as appropriate. The categorical variables were tested by chi-square test. The SPSS software (version 16.0), Microsoft Excel 2003, and the software packages in RStudio were used as the analysis and mapping software. A two-tailed test result with $p < 0.05$ was considered statistically significant. Other statistical methods were noted in the figure or figure legends.

Results

1. **Serum protein levelsin CRCs**

After screening according to the inclusion criteria and exclusion criteria, 398 cases of CRC patients were included in the retrospective study. Age-matched healthy volunteers from the physical examination center were selected as the control group. The clinical characteristics of subjects are shown in **Table 1**. There was no significant difference between the CRC patients and healthy volunteers in sex, age, BMI, personal habits including smoking and drinking, chronic diseases including diabetes and hypertension, and some serum indicators including hemoglobin, alanine transaminase (ALT), glutamic oxaloacetic transaminase (GOT), creatinine, and triglyceride. Serum nutrition-related indicators including total protein and albumin in CRC group were lower than those in control group (p□0.05). Other serum biochemical indicators such as high density lipoprotein (HDL), low density lipoprotein (LDL), and Apolipoprotein A (apoA) were also statistically different between these two groups.

Clinically, serum albumin level is an objective index for nutritional assessment. HDL, LDL and Apo A are not used as an indicator of nutritional assessment. We further analyzed the nutrition-related indicators including total protein, albumin, and globulin, and the ratio of albumin and globulin in CRC patients in terms of gender, pathological stage, and cancer site. As shown in **Table 2,** there was no significant difference in the serum nutrition-related indicators at different pathological stages and between males and females. There was no significant difference in the globulin levels and in the ratio of albumin and globulin between the colon cancers and rectal cancers, but the total protein and albumin in colon cancer was lower than that in rectal cancer (p□0.05).

2. **Association of gut microbes with different serum albumin levels in CRCs**

A total of 30 cases of CRC patients were included to detect the gut microbiota. These patients were divided into three groups according to albumin levels. F0, F1, and F2 represent ranges of albumin values of over 40 g/L, 35~40 g/L and below 35 g/L, respectively. As shown in **Table 3**, there was no significant difference among these three groups in sex, age, BMI, and some serum indicators including hemoglobin, ALT, GOT, creatinine, triglyceride, HDL, LDL, and apolA.

The gut bacteria were detected to compare the diversity among the three groups. As shown in **Supplement material-Figure S2**, panels A, B, and C show the Chao1 curves, Shannon curves, and Simpson curves, respectively. There is no statistical difference (Kruskal test) between Chao1 indexes, Shannon indexes, and Simpson indexes among the three groups.

The community structure of gut bacteria among the three groups was analyzed. **Figure 1** shows the plot of community structure among the three groups. The stacked bar graph in **Panel A of figure 1** shows the relative abundance of gut bacteria at the genus level for each sample. Despite the large variation of the bacteria in each sample, the bacteria that made up the majority of stool samples in CRC patients, in turn, included *Bacteroides, Escherichia/Shigella, Prevotella, Ruminococcaceae, Faecalibacterium, Streptococcus, Roseburia, Parabacteroides, Lachnospiraceae,* and *Clostridium XlVa.* The taxonomic tree heatmap in **Panel B of figure 1** shows the composition and proportion of microorganisms at different taxonomic levels including phylum, class, order, family, and genus. The outermost layer indicates annotated genus. The orange circles indicate that the abundance of *Sutterella* (phylum__*Proteobacteria*, class__*Betaproteobacteria*, order__*Burkholderiales*, family__*Sutterellaceae*, genus__*sutterella*) is higher in the CRCs with the lower serum albumin level (Kruskal test). The enterotype map in **Panel C of figure 1** represents the correlation and contribution of the bacteria to different groups. Orange area, cyan area, and purple area represents F0 group, F1 group, and F2 group, respectively. The result shows that *Burkholderia, Grenulicatella, Stenotrophomonas, Collinsella, Actinomyces, Oribacterium, Delftia,* and *Parasutterella* are closely related to F0 group. *Ruminococcus, Coprococcus,* and *Paraprevotella* are closely related to F1 group. *Prevotella, f_Porphyromonadaceae, Alloprevotella, Methanobrevibacter, Porphyromonas, Coprobacter, Eggerthia, Lactobacillus, Turicibacter, Pseudoflavonifractor, Campylobacter, Sutterella, and Oscilfibacter* are closely related to F2 group (Monte-Carlo simulation).

The gut fungus were detected to compare the diversity among the three groups. The **Supplement material-Figure S3** shows the alpha diversity analysis of gut fungus among the three groups including F0, F1, and F2. Panels A, B, and C shows the Chao1 curves, Shannon curves, and Simpson curves, respectively. There is no statistical difference (Kruskal test) between Chao1 indexes, Shannon indexes, and Simpson indexes among the three groups.

Figure 2 shows the community structure of gut fungus among the three groups. The stacked bar graph in **Panel A of figure 2** shows the relative abundance of gut fungus from the stool samples of CRC patients at the genus level. The fungus that can be successfully compared by the relative database in the top 10

include *Saccharomyces, Saccharomycetales, Humicola, Candida, Rhodotorula, Ascomycota, Penicillium, Malassezia, Filobasidium*, and *Debaryomyces*. The taxonomic tree heatmap in **Panel B of figure 2** shows that there were statistically significant differences in the abundance of fungus including *Agaricomycetes (p__Basidiomycota, c__Agaricomycetes, o__Agaricomycetes_unidentified, f__Agaricomycetes_unidentified_1, g__Agaricomycetes), Simplicillium (p__Ascomycota, c__Sordariomycetes, o__Hypocreales, f__Cordycipitaceae, g__Simplicillium), Sclerotiniaceae (p__Ascomycota, c__Leotiomycetes, o__Helotiales, f__Sclerotiniaceae, g__Sclerotiniaceae), and Exophiala (p__Ascomycota, c__Eurotiomycetes, o__Chaetothyriales, f__Herpotrichiellaceae, g__Exophiala)* among the three groups (Kruskal test). The enterotype map in **Panel C of figure 2** represents the correlation and contribution of the gut fungus to different groups. Orange area, cyan area and purple area represent F0 group, F1 group, and F2 group, respectively. The result shows that *Simplicillium, Bionectria, Verticillium, Hannaella, Davidiella, Thanatephorus, Acrostalagmus, Devriesia, Malassezia, Cyberlindnera, Trichocomaceae, and Asterotremella* are closely related to F0group. *Eurotiomycetes* and *Aspergillus* are closely related to F1 group. *Bionectraceae, Magnusiomyces, Sarocladium, Phoma. Kodamaea, Pichia, Pseudogymnoascus, Incertae_sedis, Trichosporon, Geotrichum, Penicillium, Auricularia,* and *Pleosporaceae* are closely related to F2 group (Monte-Carlo simulation).

3. **Association of gut microbial metabolites with different serum albumin levels in CRCs**

As shown in **Table 4**, there was no significant difference among these three groups with respect to sex, age, BMI, and some serum indicators including hemoglobin, ALT, GOT, creatinine, triglyceride, HDL, LDL, and apolA. A total of 124 microbial metabolites in 10 classes extracted from stool samples of CRC patients were quantified in the present study. The list of microbial metabolites is showed in **Supplement material-table S1.**

Figure 3 shows the relative proportion of gut microbial metabolites in each example. The stacked bar on the left represents each sample, and the stacked bar on the right represents a set of the same group. P**anel A in Figure 3** shows the proportion of gut microbial metabolites in the detected samples at class level. The results show that the gut microbial metabolites accounting for the top three include amino acids, cinmic acids, and fatty acids at class level. P**anel B in Figure 3** shows the proportion of gut microbial metabolites in the absolute quantitative top 30. The gut microbial metabolites from CRC patients in the top 10 including acetic acid, L-methionine, 3-hydroxybutyric acid, L-glutamic acid, L-lysine, glutathione, glyceric acid, oxoadipic acid, L-homoserine, and gamma-aminobutyric acid. The heatmap in **Figure 4** shows that the gut microbial metabolites had statistically significant differences among the three groups (Kruskal test). The **panel A** and **panel B in Figure 4** shows the concentration of gut microbial metabolites from each sample and each group, respectively. The results show that the stool samples in CRC patients with the lower serum albumin level had an increase in the concentration of gut microbial metabolites including 2-hydroxybutyric acid, 2-phenylglycine, D-2-hydroxyglutaric acid, glycine, L-asparagine, behenic acid, oxoglutaric acid and succinic acid, and a decrease in the concentration of gut microbial metabolites including L-norleucine, salicyluric acid, isocitric acid, and glycine.

Discussion

In the present study, serum albumin levels in CRC patients were retrospectively analyzed and compared with those of healthy controls. We found that serum albumin levels decreased in CRC patients. The total protein and albumin in colon cancer was lower than that in rectal cancer. The abundance of *Sutterella* is higher in the CRCs with the lower serum albumin level. There were statistically significant differences of some gut fungus including *Agaricomycetes, Simplicillium, Sclerotiniaceae, and Exophiala* in different serum albumin levels. The concentration of gut microbial metabolites including 2-hydroxybutyric acid, 2-phenylglycine, D-2-hydroxyglutaric acid, glycine, L-asparagine, behenic acid, oxoglutaric acid and succinic acid were increased in CRC patients with the lower serum albumin level. And some microbial metabolites including L-norleucine, salicyluric acid, isocitric acid, and glycine were decreased.

Some factors are considered to cause errors in the detection results of intestinal microorganisms. Therefore, patients with known complications such as diabetes, hypertension, smoking, and drinking history over the course of 1 year were excluded. In addition, excluded DNA extraction failure and those that did not meet quality control standards in the process of measuring gut microbiome. Finally, 30 cases were included in the study of microorganisms and 56 cases were including in microbial metabolites. The CRCs patients were divided into three groups according to albumin levels, including F0(or M0) for over 40 g/L, F1(or M1) for 35–40 g/L, and F2(or M2) for below 35 g/L. According to the reference value range of clinical serological indicators in the clinical laboratory department of the hospital, serum albumin less than 40 g/L was considered as hypoproteinemia. The guidelines for nutritional therapy for cancer patients are not clearly defined. Serum albumin less than 35 g/L was used as the reference standard for hypoproteinemia in some studies of colorectal cancer patients with hypoproteinemia[20, 21]. Taking into account the retrospective analysis of serum albumin levels in CRC and the balance of the number of patients included in the group. Therefore, this grouping criteria were selected.

Most previous studies have focused on the effects of high-protein diets on gut microbes[13, 22]. Protein ingested by the human body is first broken down into amino acids, converted into other metabolites by transamination or excreted by deamination and oxidative decomposition, a process known as nitrogen balance[23–25]. Nitrogen balance determines that a high-protein diet does not change the body's serum protein and albumin level. Thus, whether a high-protein diet has a positive or negative effect on the body is debatable. However, the negative nitrogen balance caused by cancers, especially the malignant tumors in the digestive tract, leads to malnutrition, having a negative impact on health[26]. Serum total protein mainly includes globulin and albumin, and the level of total protein and albumin reflects the nutritional status of patients. Therefore, studying the relationship between negative nitrogen balance and gut microbes is essential.

The present study found that serum albumin levels decreased in CRC patients. This predictable result has been confirmed by other studies[27, 28]. However, we also found that the total protein and albumin in colon cancer was lower than that in rectal cancer. Besides the difference in the anatomical site, there could be other factors, such as bleeding, intestinal obstruction and tumor growth rate, contributing to

hypoproteinemia. Factors leading to hypoproteinemia and effect of hypoproteinemia on the prognosis of CRC need further investigation.

We found that the top three bacteria in the stool sample from CRCs were *Bacteroides, Escherichia/Shigella*, and *Prevotella.* The top three bacteria association with CRC has also been previously reported. For instance, several studies suggested that *Bacteroides* are the bacteria that induce CRC[29, 30]. Tingting, et al. reported that *Escherichia/Shigella* were present at higher concentrations in the stool of CRC patients that in stools of healthy volunteers[31]. A review showed that gut *Prevotella* was correlated with diets and non-communicable diseases including CRC[32].

The results in the present study showed that the higher abundance of *Sutterella* is correlated with lower serum albumin level in CRCs. *Sutterella* is a commensal that can adhere to intestinal epithelial cells and promote inflammation and has been associated with human diseases, such as inflammatory bowel disease (IBD) [33]. IBD induces CRC through repeated cycles of epithelial injury and repair[34]. However, the relationship between hypoproteinemia and *Sutterella* in CRC remains unclear. The correlation between nutritional status and the species level and subspecies level with regard to the genus *Sutterella* is worth further study.

In recent years, more attention has been paid to the study of gut fungus[35]. Our results show that the fungus *Saccharomyces, Saccharomycetales*, and *Humicola* accounted for the top three in stool samples of CRC patients. Glinka, et al. reported a result consistent with our study that the abundance of *Saccharomyces genus* was increased in gastrointestinal malignancies[36]. Heat-killed form of *Saccharomyces cerevisiae* acted as a probiotic and caused apoptosis in colon cancer cells[37]. *S*uch reports with regard to *Humicola* in cancer are rare. Harsimran, et al. reported that they isolated *Ochroconis humicola* from the tissue of esthesioneuroblastoma[38], but not CRC. There was rarely reported on the role in nutrition status. fungus, as an important component of microorganisms, should not be overlooked in CRC research. We're still in the infancy stage of studying the complex relationship among fungus, CRC, and nutrition.

There are multiple studies on the microbial metabolites short chain fatty acids (SCFAs) in relation to CRC and nutrition[9,39–41]. The metabolomics method including 124 microbial metabolites allows for a more comprehensive assessment of stool samples from CRC patients. The results showed that some microbial metabolites, such as acetic acid, L-methionine, 3-hydroxybutyric acid, L-glutamic acid, and L-lysine, were relatively higher in the fecal samples from CRC patients than healthy subjects. It has been studied extensively that acetic acid, as one of the components of SCFAs, functions in the suppression of CRC [42]. The recombinant L-methionine gamma-lyase from *Brevibacterium aurantiacum* can eliminate L-methionine and induce growth arrest and death of CRC cells[43]. Yui, et al. reported that 3-hydroxybutyric acid can reprogram energy metabolism during glucose starvation and inhibit the proliferation of CRC cells[44]. The supplementation of glutamic acid can decrease the risk of CRCs[45, 46]. We found some characteristic gut microbial metabolites to be increased or decreased in CRC patients correlating with the

lower serum albumin level in the present study. Due to the lack of similar reports and the insufficient sample size in this study, the results need to be verified by multi-center experiments with larger sample size.

It is of great significance to study the association between nutritional status and gut microbes and microbial metabolites in CRC. Firstly, it may provide a novel direction to translational medicine in the assessment of nutritional status from the perspective of microbiology. Secondly, stool samples have the advantages of no innovation and easy access. The correlation analysis of gut bacteria and fungus with different albumin levels may provide some basis for building a network of interactions between microbes and nutrients. Specifically, the network contains the relationship between the microbes including diversity and community structure of bacteria and fungus, quantification of microbial metabolites and nutrients including proteins, fats, and carbohydrates. The dynamic monitoring of characteristic microbes and microbial metabolites in the stool may provide a simple and rapid early warning and screening method for malnutrition. Thirdly, some means of intervention, such as fecal transplantation, oral administration of microorganisms, supplementation of microbial metabolites, and dietary choices may provide alternative treatments and auxiliary treatment for patients with malnutrition.

We acknowledge the shortcomings of this study. Considering that the investigation may be influenced by a variety of factors such as geographical location, nosocomial infection, or pollution contamination in the fecal collection process, we enrolled CRC patients from the same region and ethnic group to minimize the influence of diet on intestinal microorganisms. More, the strict detection standards, sample collection standards, inclusion and exclusion criteria were conducted in the present study. However, the influence of individual dietary habits and life style on microecology is inevitable. In addition, many ITS sequences cannot be annotated. The 125 microbial metabolites are not the full spectrum of microbial metabolites. The investigation needs to be further in-depth and verified.

The interrelation and interactions between microbes and metabolites in the intestinal tract are very complex. There are obvious defects in using single microbes or metabolites to predict the nutritional status. Our future plans include acquiring larger data of microbiology and microbial metabolomics by including polycentric, multi-regional samples. A more complete and scientific nutritional status assessment model will be developed by applying machine learning models, such as neural network model or random forest model. These larger studies should provide clinical guidance for nutritional assessment and intervention.

Conclusions

The serum total protein and serum albumin levels in CRC group were lower than those in healthy people. We determined characteristic gut microbes and microbial metabolites that correlated with the different serum albumin levels in CRCs, and our findings further support the hypothesis that intestinal microorganisms and microbial metabolites may be related to host nutrient metabolism. It may provide novel ideas for basic research and clinical application.

List Of Abbreviations

Abbreviation	Definition
CRC	Colorectal Cancer
GS	Gas Chromatography
MS	Mass Spectrometer
MDT	Multidisciplinary comprehensive treatment
OTU	Operational taxonomic unit
SD	Standard deviation
AJCC	American Joint Committee on Cancer
NCCN	National Comprehensive Cancer Network
UICC	International Cancer Control
PCR	Polymerase Chain Reaction
rDNA	ribosomal Deoxyribonucleic Acid
MCF	Methyl chloroformate
TOFMS	Time-of-Flight Mass Spectrometry
EI	Electron Ionization
FBG	Fasting Blood Glucose
OTU	Operational Taxonomic Unit
ALT	Alanine transaminase
GOT	Glutamic oxaloacetic transaminase
HDL	High density lipoprotein
LDL	Low density lipoprotein
apoA	Apolipoprotein a
BFT	Bacteroides fragilis toxin
IBD	inflammatory bowel disease

Declarations

Ethics approval and consent to participate

The clinical protocols involving the patients and the informed consent form were approved by the Chinese Clinical Trial Registry (http://www.chictr.org.cn, No. ChiCTR1800018908) and Ethics Committee of Huzhou Central Hospital (No. 201601022, No. 20190204).

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during the current study are not publicly available but obtained from corresponding authors on reasonable request.

Competing interests

The authors declare that no conflicts of interest exist.

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Authors' contributions

All authors participated in the conception and design of the study;

Conceived and drafted the manuscript: Han Shuwen and Yang Xi;

Performed the experiments: Wu Wei, Da Miao ;

Collected the basic patient information, clinical indicators, and imaging data: Zhuang Jing and Xu Jiamin;

Analyzed the data: Zhou Qing and Liu Jin ;

Wrote the paper: Han Shuwen and Yang Xi;

All authors read and approved the paper.

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Tables

Table 1. Characteristics of the Subjects with Regard to Clinical Investigation

	Colorectal Cancer group	Healthy Control group	p value
Cases, n	398	398	/
Males, n	236	231	0.669
Age, years	64.59±9.13	64.21±8.89	0.555
BMI, kg/m^2	22.39±3.02	22.45±2.84	0.793
Long-term smoking history, n	98	88	0.373
Long-term drinking history, n	64	63	0.920
Known diabetes, n	26	20	0.448
Known hypertension, n	98	88	0.451
Hemoglobin (g/L)	122.12±21.15	124.71±20.93	0.082
Alanine transaminase (U/L)	17.09±9.93	16.85±9.72	0.731
Glutamic oxaloacetic transaminase (U/L)	21.56±6.50	21.62±7.23	0.890
Total protein (g/L)	65.03±6.58	70.73±8.32	0.001
Albumin (g/L)	39.25±4.29	44.97±3.34	0.001
Globulin (g/L)	25.78±4.19	25.75±7.42	0.952
Creatinine (μmol/L)	68.95±16.60	68.86±16.73	0.941
Triglyceride (mmol/L)	1.35±0.66	1.26±0.59	0.054
HDL (mg/dL)	44.20±12.24	96.29±26.07	0.001
LDL (mg/dL)	100.41±27.11	20.41±15.89	0.001
Apol a (mg/dL)	28.56±28.22	20.42±15.89	0.001

The clinical stages conformed to the criterion of CRC according to the UICC/AJCC TNM staging system (Version 8, 2017). Smoking and drinking history over the course of 1 year were collected. Serum nutrition-related indicators from CRC patients before treatments including chemotherapy, radiation, and surgery were collected. Chi-square test and student t-test was used for counting data comparison and measuring data comparison, respectively.

Table 2. Serum nutrition-related indicators in CRCs with respect to gender, pathological stages, and cancer sites

Groups	Subgroups	Cases	Total protein	Albumin	Globulin	Albumin/ Globulin
Reference ranges		—	65-85 g/L	40-55 g/L	20-40 g/L	1.20-2.40
Colorectal cancer		398	65.03±6.58	39.25±4.29	25.78±4.19	1.56±0.28
Pathological stages	Stage I	64	63.45±5.01	38.67±3.85	24.79±3.69	1.60±0.31
	Stage II	270	64.87±6.67	39.24±4.43	25.63±3.95	1.56±0.26
	Stage III	64	67.25±7.05	39.84±4.08	27.41±5.17	1.50±0.31
	F value	—	5.697	1.194	6.987	1.910
	p value	—	0.153	0.175	0.186	0.068
Sex	Male	236	64.61±6.67	38.96±4.28	25.65±4.05	1.55±0.26
	Female	162	65.63±6.41	39.66±4.28	25.97±4.39	1.57±0.30
	t value	—	1.519	1.587	0.759	0.664
	p value	—	0.130	0.113	0.448	0.507
Sites	Colon	188	64.34±6.47	38.77±4.39	25.57±3.93	1.55±0.27
	Rectum	210	65.64±6.63	39.67±4.17	25.96±4.42	1.57±0.28
	t value	—	1.978	2.118	0.940	0.678
	p value	—	0.049	0.035	0.348	0.498

The serum nutrition-related indicators in CRCs with respect to gender, pathological stages, and cancer sites were investigated and analyzed. The clinical stages conformed to the criterion of CRC according to the UICC/AJCC TNM staging system (Version 8, 2017). Serum nutrition-related indicators from CRC patients before treatments including chemotherapy, radiation, and surgery were collected. Independent

sample t- test was used for comparison of measurement data between the two groups, and SNK test was used among the three groups.

Table 3. Characteristics of the study participants with regard to microbiota investigation

	F0	F1	F2	p value
Cases, n	8	14	8	/
Males, n	4	7	6	0.474
Age, years	64.13±9.73	64.71±6.58	65.00±8.45	0.975
BMI, kg/m^2	21.52±2.34	21.78±3.60	22.60±1.83	0.736
Hemoglobin(g/L)	120.88±15.64	115.07±20.24	109.13±25.85	0.537
ALT(U/L)	24.89±23.94	14.00±6.12	15.95±6.96	0.193
GOT(U/L)	22.61±11.77	19.69±4.78	19.65±5.31	0.632
Total protein(g/L)	70.81±4.23	62.26±3.26*	55.34±5.22#	0.001
Albumin(g/L)	43.98±1.67	37.61±1.50*	33.11±1.40#	0.001
Globulin(g/L)	26.84±2.65	24.64±2.90	22.21±4.46	0.033
Creatinine(μmol/L)	62.90±8.12	63.30±12.64	80.01±32.50	0.123
Triglyceride(mmol/L)	1.11±0.31	1.24±0.61	1.19±0.60	0.859
HDL (mg/dL)	52.25±10.39	46.14±15.85	39.95±10.24	0.198
LDL (mg/dL)	84.72±35.99	99.55±25.95	83.90±17.10	0.323
ApolA(mg/dL)	37.39±28.20	27.97±22.63	33.80±23.06	0.667

F0, F1, and F2 represent ranges of albumin values of over 40 g/L, 35~40 g/L and below 35 g/L, respectively. Serum nutrition-related indicators from CRC patients before treatments including chemotherapy, radiation, and surgery were collected. Patients with known complications such as diabetes, hypertension, smoking, and drinking history over the course of 1 year were excluded. Three groups of measurement data were compared using SNK test.(* means that $p<0.05$, compared to the F0 group, # means that $p<0.05$, compared to the F1 group.)

Table 4. Characteristics of the study participants with regard to microbial metabolites investigation

	M0	M1	M2	p value
Cases, n	14	28	14	
Males, n	5	14	9	0.215
Age, years	63.52±9.00	64.95±6.59	66.53±6.69	0.442
BMI, kg/m^2	21.87±2.60	22.21±3.64	21.70±2.69	0.823
Hemoglobin (g/L)	122.26±12.58	118.43±18.88	109.47±21.11	0.069
Alanine transaminase (U/L)	19.77±11.61	14.89±5.37	17.70±7.78	0.161
Glutamic oxaloacetic transaminase (U/L)	22.87±9.99	20.70±4.38	22.39±6.47	0.412
Total protein (g/L)	71.07±4.22	62.34±3.56*	57.16±5.95$^{\#}$	0.001
Albumin (g/L)	43.67±2.10	37.76±1.52*	32.87±1.35$^{\#}$	0.001
Globulin (g/L)	27.39±3.19	24.57±3.10	24.28±5.28	0.008
Creatinine (μmol/L)	64.64±8.31	65.02±17.82	67.26±21.90	0.904
Triglyceride (mmol/L)	1.26±0.59	1.26±0.57	1.00±0.45	0.210
HDL (mg/dL)	49.14±10.07	45.94±14.54	42.50±9.47	0.232
LDL (mg/dL)	106.43±31.61	10005±24.51	102.14±31.79	0.789
Apolipoprotein a (mg/dL)	31.52±26.45	30.64±34.28	37.78±24.90	0.686

M0, M1 and M2 represent ranges of albumin values of over 40 g/L, 35~40 g/L and below 35 g/L, respectively. Serum nutrition-related indicators from CRC patients before treatments including chemotherapy, radiation, and surgery were collected. Patients with known complications such as diabetes, hypertension, smoking, and drinking history over the course of 1 year were excluded. Three groups of measurement data were compared using SNK test.(* means that $p<0.05$, compared to the M0 group, $^{\#}$ means that $p<0.05$, compared to the M1 group.)

Figures

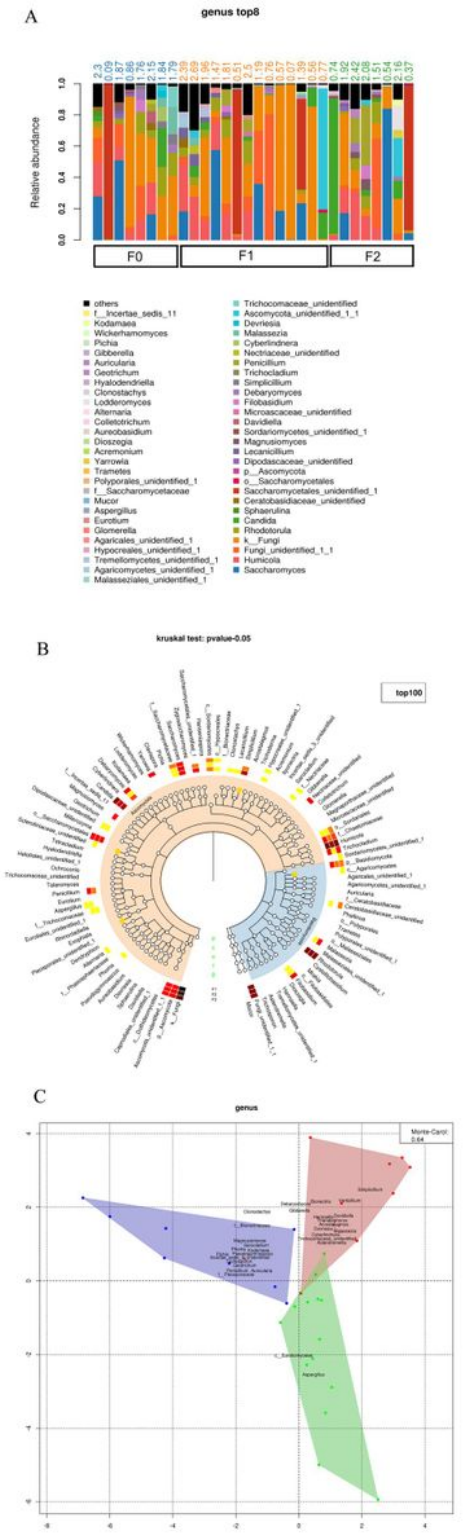

Figure 1

The community structure of gut bacteria with different serum albumin levels in CRCs The panel A in Figure 3 shows relative abundance of the gut bacteria at the genus level in CRCs with different serum albumin levels. The panel B in Figure 3 shows composition and proportion of bacteria at different taxonomic levels in CRCs with different serum albumin levels. The panel C in Figure 3 shows association of gut bacteria with serum albumin levels in CRCs.

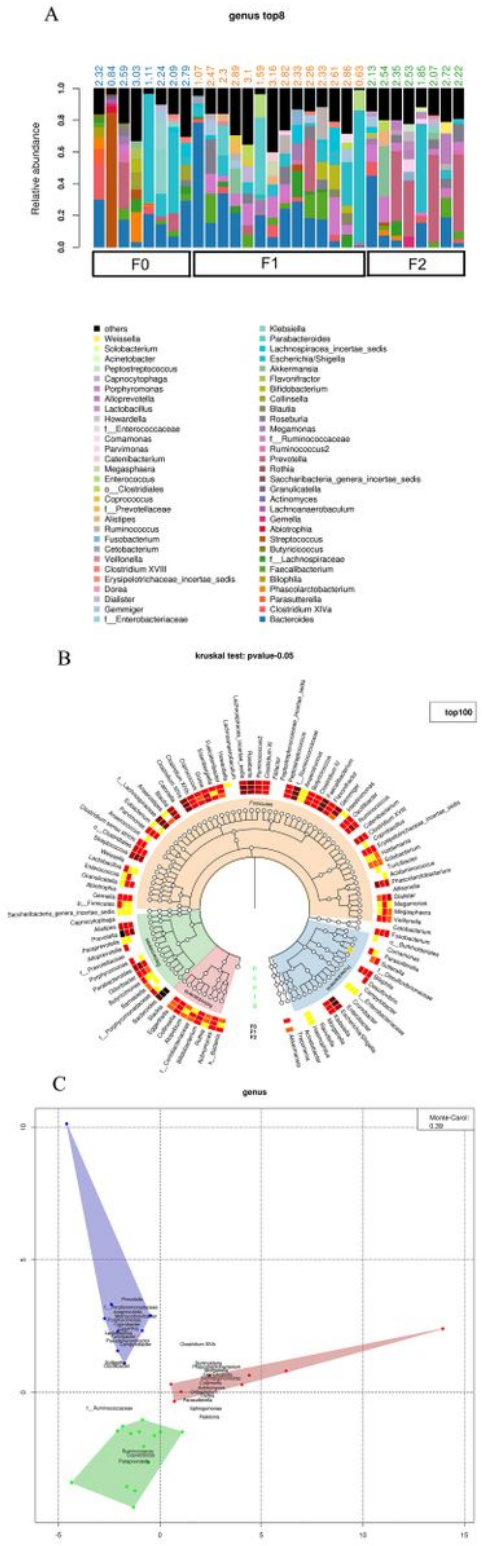

Figure 2

The community structure of gut fungus with different serum albumin levels in CRCs The panel A in Figure 2 shows relative abundance of the gut fungus at the genus level in CRCs with different serum albumin levels. The panel B in Figure 2 shows composition and proportion of fungus at different taxonomic levels in CRCs with different serum albumin levels. The panel C in Figure 2 shows association of gut fungus with serum albumin levels in CRCs.

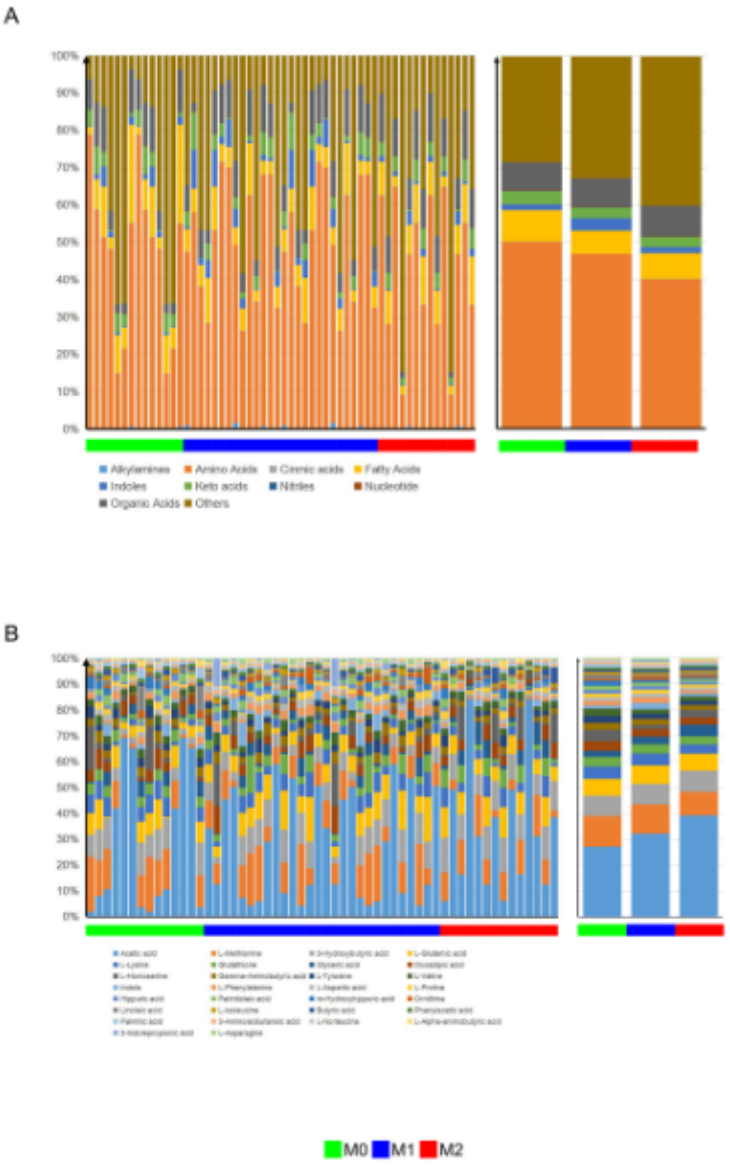

Figure 3

The proportion of gut microbial metabolites from CRCs A total of 56 CRC patients were enrolled to determine the gut microbial metabolites. Patients were divided into three groups according to albumin levels. M0, M1 and M2 represent ranges of albumin values of over 40 g/L, 35~40 g/L and below 35 g/L, respectively. In total, 124 microbial metabolites in 10 classes extracted from stool samples of CRC patients were quantified. The panel A in Figure 3 shows the proportion of gut microbial metabolites at class level. The panel B in Figure 3 shows the proportion of gut microbial metabolites in the absolute quantitative top 30.

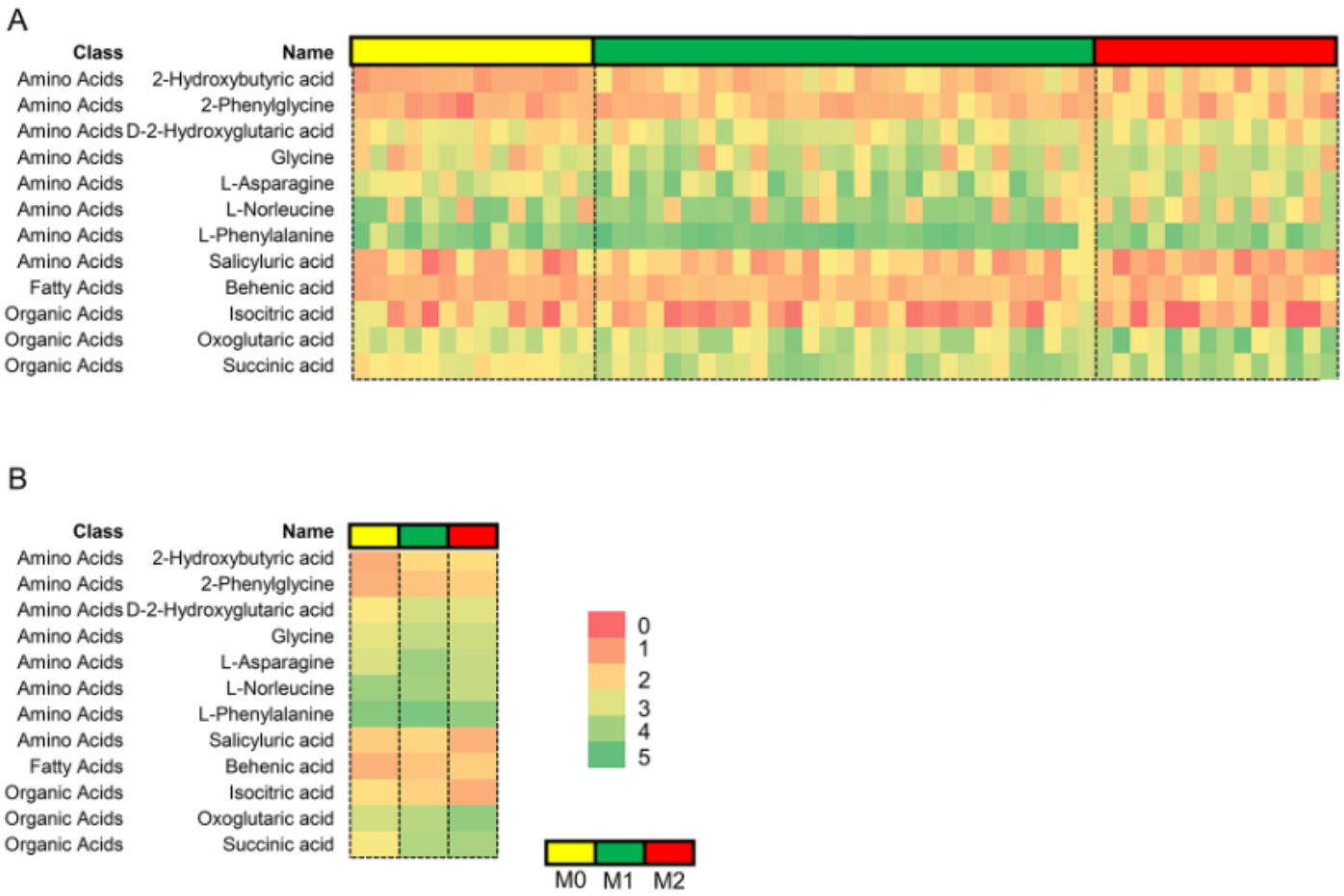

Figure 4

Association of the concentration of microbial metabolites with serum albumin levels in CRCs The GC/TOFMS analysis was used to determine the amount of microbial metabolites per milliliter of the stool samples from 56 CRC patients. Patients were divided into three groups according to albumin levels. M0, M1 and M2 represent ranges of albumin values of over 40 g/L, 35~40 g/L and below 35 g/L, respectively. The figure shows the gut microbial metabolites with statistically significant differences among the three groups (Kruskal test). The logarithm base 10 of the sample concentration (ng/ml) was used to generate a heat map. Panel A and panel B show the gut microbial metabolites from each sample and each group, respectively.

Supplementary Files

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