Effect of Disordered Gut Microbiota on Serum Metabolome Alterations in Colorectal Tumor-Bearing Mice Under The Intervention of Fufangchangtai

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Research

Keywords: Colorectal cancer, Fufangchangtai, Gut microbiota, Serum metabolome

Posted Date: April 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-426536/v1

Effect of disordered gut microbiota on serum metabolome alterations in colorectal tumor-bearing mice under the intervention of *Fufangchangtai*

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Abstract:

Background: The occurrence and development of colorectal cancer is related to the compositional and functional variation of gut microbiota. Compared with healthy people, gut microbiota of patients with colorectal cancer is in disorder. Most traditional Chinese Medicine is effective by oral administration and has both anti-tumor effect and enteric microecological regulative effect. However, whether the dysbiosis of gut microbiota under tumor burden affects the serum metabolome of human body that related to traditional Chinese medicine is unclear. In this study, *Fufangchangtai*(FFCT) was chosen to be the model prescription to explore the correlation between gut microbiota and the serum metabolism related to FFCT in anti colorectal cancer treatment.

Results: The gut microbiota between colorectal tumor-bearing mice and healthy mice were determined by 16S rRNA gene sequencing, showing quite differences between the two groups and suggesting that *Firmicutes*, *Deferribacteres*, *Bacteroidetes* and *Proteobacteria* were marked differential intestinal bacteria. The alternations in serum metabolome in the FFCT-treating tumor-bearing mice and simple FFCT-treating mice were detected using Liquid chromatogragh-mass spectrometer (LC/MS), showing significant differences between the two groups as well. Metabolites of FFCT like Citric acid, （±）12-HEPE, Cycloartanyl ferulate were much more in simple FFCT-treating mice, indicating that the present of tumor could affect the absorption and metabolism of FFCT. Additionally, these differential metabolites of FFCT involved in multiple pathways including the Alanine, aspartate and glutamate metabolism, Central carbon metabolism in cancer, Biosynthesis of amino acids. Different doses of FFCT were given to the tumor-bearing mice through oral administration, and the results of gut microbiota 16S rRNA gene sequencing showing that FFCT-treating groups has higher abundance of *Firmicutes*, *Turicibacter* and *Roseburia* than tumor-bearing group, moreover, the abundance of these bacteria was positively correlated to the drug concentration. *Firmicutes* and *Bacteroidetes* in FFCT-treating groups showed a similar trend with Healthy group, indicating the modulation of FFCT on gut microbiota of colorectal tumor-bearing mice.

Conclusions: Collectively, we concluded that the dysbiosis of gut microbiota in tumor-bearing mice could affect the serum metabolome of human body that related to FFCT, and FFCT could correct the gut microbiota of colorectal tumor-bearing mice. It was pointed out that the GM should be concerned during the therapy of FFCT. The more healthier intestinal microenvironment was conductive to the better clinical curative effect.

Keywords: Colorectal cancer, *Fufangchangtai*, Gut microbiota, Serum metabolome

1 Introduction

Colorectal cancer (CRC) is a malignant tumor that occurs to the colon or rectum. Although the death rate of CRC is decreasing continuously due to the population screening and endoscopic surveillance, it's incidence rate and mortality rate is still the third worldwide[1]. Gut microbiota (GM), which is called 'the new virtual metabolic organ'[2], plays a vital role in stimulating the digestion[3], producing nutrients [4], and promoting mucosal barrier immunity[5]. Once GM is out of balance, systematic diseases including carcinoma will occur. Many evidences indicated that there was a correlation between the development of CRC and dysbiosis of GM. Bacterial genes obtained by pyrosequencing of stool samples of healthy people and patients with CRC were subjected to the Principal Component Analysis (PCA) test. According to the PCA analysis, there

were trends towards differences in the dominant and subdominant families of bacteria in CRC individuals and normal individuals[6]. GM initiates CRC by building tumoral microenvironment and inducing epithelial DNA damage and tumorigenesis, according to the 'diver-passenger' theory[7]. Meanwhile, GM also affects the immunity and prognosis of CRC patients by producing immunostimulatory and/or immunosuppressive cytokines such as IL-17A and IL-9[8]. Studies showed that tumor characterized by IL-17 expressing T cells, FOXP3hi Tregs and immunosuppressive myeloid populations were associated with worse clinical outcome[9].

There are many treatments for CRC, including radical surgical, chemotherapy, radiotherapy, targeted therapy, immunotherapy, etc. Correspondingly, these treatments have their own limitations and bring about many adverse reactions. Therefore, Traditional Chinese medicine (TCM) plays an important role in the prevention of tumor recurrence and metastasis due to its effect on synergy and attenuation. Chinese decoction such as Xiaoyaosan successfully reduced the tumor volume and tumor weight in mice with CRC xenografts and prolonged the overall survival time through a 21-days intragastrical treatment[10]. Herb such as Matricaria chamomilla could act as a potent single component against DMH-induced CRC by modulating the Wnt pathway[11]. *Fufangchangtai* (FFCT) is a prescription that has an effect on treatment of CRC by enhancing the body's immune, improving the patient's quality of life and down-regulating the side effects, which had been proved by clinical observation [12].

Most TCM is taken orally and absorbed into the blood through the gastrointestinal tract, thus exerting its efficacy. Therefore, TCM can reverse or mitigate varied compositional dysbiosis of GM associated with many diseases besides improving the pathological symptoms[13]. Study showed that *Berberine* rescued *F. nucleatum*-induced colorectal tumorigenesis by modulating the tumor microenvironment and blocking the activation of tumorigenesis-related pathways[14]. About TCM formula, Yi-Yi-Fu-Zi-Bai-Jiang-San (YYFZBJS) blocked tumor initiation in treating CRC by mediating Tregs and regulating the natural GM of Apc (Min/+) mice including Bacteroides fragilis, Lachnospiraceae and so on[15]. GM harbors many types of enzymes, allowing plenty of catalytic reactions, which are involved in the biotransformation of TCM components. Though many investigations indicated that GM could interact complicatedly with TCM components, whether GM could influence the therapeutic effect of TCM on CRC still remains unknown.

Thus, there is the speculation that the dysbiosis of GM affects the serum metabolome of human body that related to TCM in treating CRC. To test the hypothesis, this study chose FFCT as the model prescription. Different doses of FFCT were given to CRC tumor-bearing mice. The 16S rRNA gene sequencing technology was applied to analysis the bacteria species in the fecal

samples, and the Liquid Chromatography Coupled to Tandem Mass Spectrometry (LC/MS) technology was applied to analysis the metabolites in the blood. The above data were analyzed to explore the correlation between GM and the metabolism of TCM in CRC treatment.

2 Materials and methods

2.1 Preparation of drugs

FFCT was formed by 6 kinds of TCM granules, including Ginseng, Membranous milkvetch root, Radix Actinidiae chinensis, August letters, Raw coix seed, Radix sophorae flavescentis, which were purchased from Jiangsu Hospital of integrated traditional Chinese and Western Medicine. The quality of formula was monitored by HPLC analysis in our previous study[16]. The formula granules were milled to powders and dissolved in warm distilled water to giving to mice.

2.2 Cell culture and injection

CT26-LUC cells were provided by Shanghai Institute of Veterinary Medicine and cultured with RPMI-1640 containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 4μg/ml puromycin dihydrochloride in an incubator. The condition of the incubator was stable, with the temperature of 37℃ and a humidified atmosphere with the 5% CO2.

2.3 Experimental Animals

Six-week-old female BALB/C mice (ethics number: SHVRI-SZ-20200420-01, SHVRI-SZ-20200720-01) were purchased from Shanghai Jiesijie Company (Shanghai, China) and housed under a specific pathogen-free (SPF) condition with 25±2℃ temperature and a 12-h light/dark cycle. The mice were freely accessed a standard rodent diet and drinking water.

2.3.1 Modeling and groups

After one week adaption of the new environment, these mice were randomly divided into six groups: the PBS group (normal mice administered with PBS, n=4), the CM group (normal mice administered with FFCT, n=5), the CT26 group (CRC tumor-bearing mice administered with PBS, n=5), the CT26-L group (CRC tumor-bearing mice administered with 0.65mg/g FFCT, n=5), the CT26-M group (CRC tumor-bearing mice administered with 1.3mg/g FFCT, n=5), the CT26-H group (CRC tumor-bearing mice administered with 2.6mg/g FFCT, n=5). The daily dosage for the CT26-M group was obtained based on the daily dosage for patients (10g/70kg) in clinical, according to the human-mouse transfer formula (Mouse dose=Human dose×9.1[17]). Before administration, CT26-LUC cells (1×10^6) were resuspended in 100μl of PBS and the suspension was inosulated subcutaneously into the flanks of mice in CRC tumor-bearing groups, while pure 100μl PBS was inosulated subcutaneously into the flanks of mice in the PBS group and the CM group. 200μl PBS or FFCT suspension was given to mice via gastric gavage, and the

administration lasted for 4 weeks.

2.3.2 Samples collection

Faecal collection: Stools were collected from the mice the day before sacrificing mice. The mice were separated in cage individually and the clean toilet paper was laid under the cage to accept their faecal pellets and absorb the urine, avoiding contamination of samples. 2-4 faecal pellets per mice were collected in 1.5ml Ep tube and stored at -80℃ until analyzed.

Blood collection: Drawing the blood from the eyeball of mice after the last oral administration 24h and keeping the blood static cool down at 4℃ for 1h. Then the serum was separated through centrifugation in 3000rpm for 5min at 4℃ and stored at -80℃ until analyzed.

2.4 GM analysis

Nucleic acid sequence of the bacterial 16S rRNA gene has been used for several decades to identify routine identification of bacterial pathogens[18].The 16S rRNA gene sequencing technology was applied to detect the V3-V4 region of bacterial species in twenty-four stool samples from five groups (PBS, CT26, CT26-L, CT26-M, CT26-H) and was entrusted to Personal Bio company.

The OMEGA Soil DNA Kit (D5625-01) (Omega Bio-Tek, Norcross, GA, USA) was used to extract the DNA from fecal samples. 16S rRNA amplicons covering variable regions V3 to V4 were generated using the forward primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Then, amplicons were sequenced using the Illumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). Sequence data analyses were mainly performed using QIIME2 and R packages (v3.2.0).

2.5 Blood metabolomics analysis

Metabolites are stable in serum and can be quantified, which presents an opportunity in monitoring disease status and exploring biomarkers to predict the efficacy of anticancer therapies[19]. The blood metabolomics of four groups (PBS, CM, CT26, CT26-H) were detected. The blood was drawn from the eyeball of mice and then stood at 4 ℃ for 1 hour. After 15min centrifugation of 3000rpm at 4 ℃, the supernatant was collected and stored in dry ice for blood mass spectrometry. Liquid chromatogragh-mass spectrometer analysis of blood was entrusted to Luming Bio company.

100 μl of serum from each sample was eddied with 10 μl internal standard (0.3 mg/ml L-2-chlorophenylalanine or 0.01mg/ml Lyso PC 17:0 dissolved in methanol) for 10 s. The

mixtures were precipitated by 300 μl mixtures of methanol and acetonitrile (2/1, v/v) and then were ultrasonicated in ice water bath for 10min, stored at -20°C for 30 min. After 10 min centrifugation (13000rpm, 4°C), 300 μl supernatant of each sample was collected and dried. The dried supernatant was resolved in 400 μl of 20% methanol (diluted by water) and then placed at -20°C for 2 h. Times up, 150 μl supernatant per sample was filtered through 0.22 μm microfilter and transferred into LC sampling vial for LC/MS analysis. Additionally, the quality control (QC) sample was created by mixing an aliquot of equal volume of each sample.

A Dionex Ultimate 3000 RS UHPLC system (Thermo Fisher Scientific, USA) coupled with Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) was used to analyze the metabolic profiling in both ESI positive and ESI negative ion modes. 2 μl prepared sample was injected into ACQUITY UPLC HSS T3 column (1.8 μm, 2.1×100mm). All samples were eluted using a linear gradient from 100% mobile phase A (0.1% formic acid in water) to 100% mobile phase B (0.1% formic acid in acetonitrile) under the condition that the flow rate was 350 μl/min and the column temperature was 45°C. Linear gradient: 0 min, 5% B; 2min, 5% B; 4min, 25% B; 8min, 50% B; 10min, 80% B; 14 min, 100% B; 15 min, 100% B; 15.1min, 5%B and 16min, 5%B. The electrospray ionization (ESI) source operating in positive and negative mode (Water, USA) was used for mass spectrometry analysis. Parameters of mass spectrometry were as follows: Capillary temperature was set at 320°C while the aux gas heater temperature was set at 350°C. Sheath gas flow rate was 35Arb and aux gas flow rate was 8Arb. The san range was from 100 to 1000 m/z.

The raw data was processed by the Progqenesis QI v2.3 software (Nonlinear Dynamics, Newcastle, UK) for baseline filtering, peak recognition, peak alignment and retention time correction. The Human Metabolome Database (HMDB), Lipidmaps (v2.3) and METLIN database and self-built database were used to identify the compounds.

3 Results

3.1 There were differences in GM between CRC tumor-bearing mice and healthy mice

There is increasing evidence that GM in CRC patients is different from that in healthy people. The Faecal microbiota of CRC patients has lower temporal stability and higher diversity[20], and an individualised oncogenic microbiome and specific bacterial species have been identified through metagenomic and meta transcriptomic studies[21]. *Streptococcus gallolyticus*, *Enterococcus faecalis* and *B. fragilis*, et al, are considered as specific pathobionts with CRC[22]. To prove the difference in GM, the faecal of CRC tumor-bearing mice were used to detect the microbiota comparing with that of healthy mice.

The GM of five groups (PBS, CT26, CT26-L, CT26-M, CT26-H) was analyzed. The

rarefaction curves of five groups (Figure 1) were close to the saturation platform at the sequencing depth of 10000, indicating that the data was sufficient enough to reflect the GM information about all the samples. The Venn diagram (Figure 2) which was plotted according to the OTUs cluster analysis showed that there were 1395 common OTUs in all five groups.

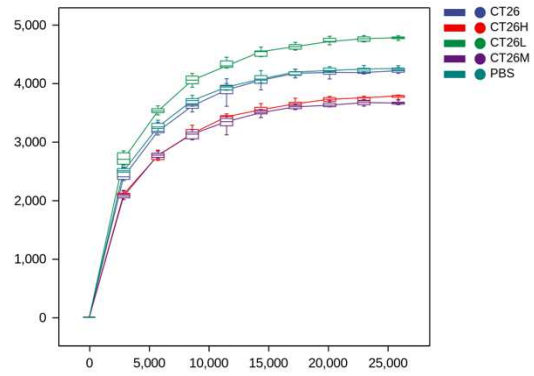


Figure 1 The rarefaction curves of each group based on Chao1

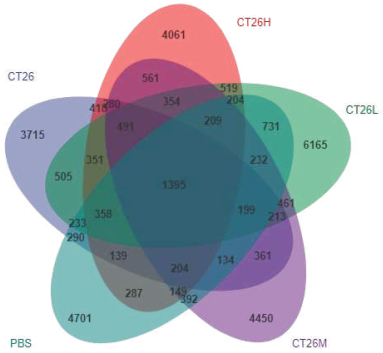


Figure 2 Venn diagram of OTUs in each group

The results of statistical analysis showed a significant difference in GM between the Tumor-bearing mice and healthy mice. The principal co-ordinates analysis (PCA) reflected the similarity in species abundance composition of two samples in the corresponding dimensions by analyzing the projection distance of the samples on the coordinate axis. As for CT26 group and PBS group, the PCA diagram (Figure 3(a)) showed that the species abundance composition of the two groups can be classified obviously. The Rank Abundance Curve consists of broken lines, and each broken line represents a group. The length of the broken line on the horizontal axis reflects the number of OTU in the specific abundance. The smoother the broken line is, the smaller difference of the OTU diversity in the community is. As for CT26 group and PBS group, the abundance grade curve (Figure 3(b)) illustrated that the Healthy Group (PBS) has higher community evenness than Tumor-bearing group (CT26). The alpha diversity analysis (Figure 3(c)) showed that there were no significant differences in Chao1, Good's coverage, Shannon, Simpson and Observed species indices between CT26 group and PBS group. However, beta diversity analysis using both unweighted and weighted UniFrac distances indicated that PBS group had higher dissimilarities among gut microbial communities than CT26 group (Table 1,2).

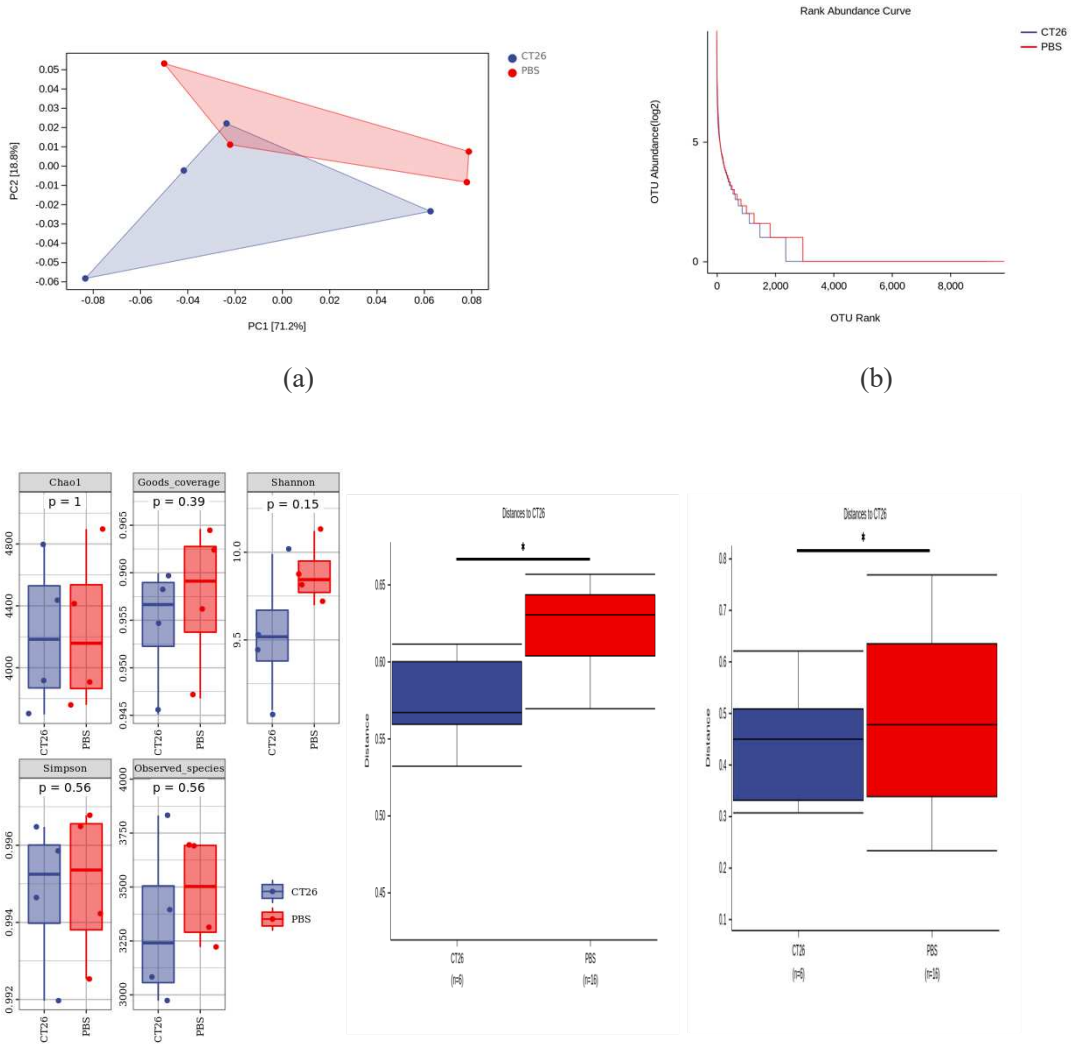
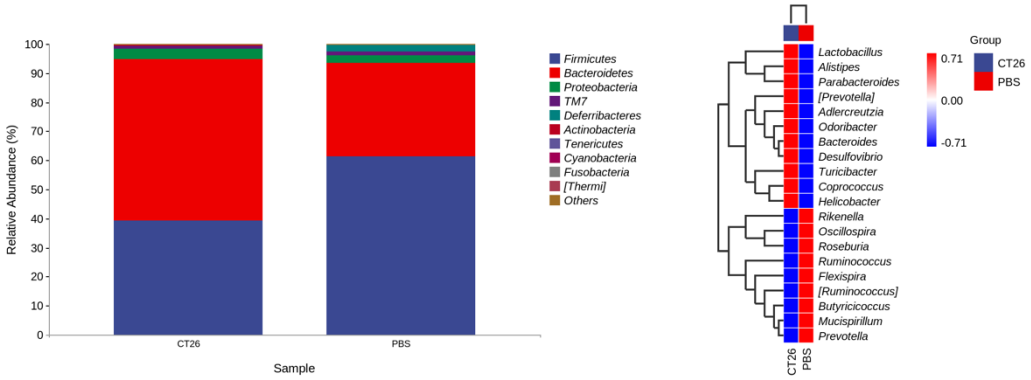


Figure 3 (a) The PCA diagram of CT26 group and PBS group. (b) The abundance grade curve of CT26 group and PBS group. (c) The differences in Chao1, Good's coverage, Shannon, Simpson and Observed species indices between CT26 group and PBS group. (d) The differences in unweighted UniFrac distance between CT26 group and PBS group. (e) The differences in weighted UniFrac distance between CT26 group and PBS group.

The taxonomic summary figure (Figure4(b)) showed that the composition of GM in the two groups (CT26 group and PBS group) was mainly characterized by the *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *TM7* and *Deferribacteres*. A marked difference between the two groups existed on the distribution of phyla. The abundance of *Firmicutes* and *Deferribacteres* in PBS group was higher than that in CT26 group, while the abundance of *Bacteroidetes* and *Proteobacteria* was lower. As showed in Figure4(b), species composition in two groups at genus level was quite different as well. Species with high expression in CT26 group were *Lactobacillus*, *Alistipes*,

Parabacteroides, *[Prevotella]*, *Adlercreutzia*, *Odoribacter*, *Bacteroides*, *Desulfovibrio*, *Turicibacter*, *Coprococcus*, *Helicobacter*. And species with low expression in CT26 group were *Rikenella*, *Oscillospira*, *Roseburia*, *Ruminococcus*, *Flexispira*, *[Ruminococcus]*, *Butyricicoccus*, *Mucispirillum*, *Prevotella*.

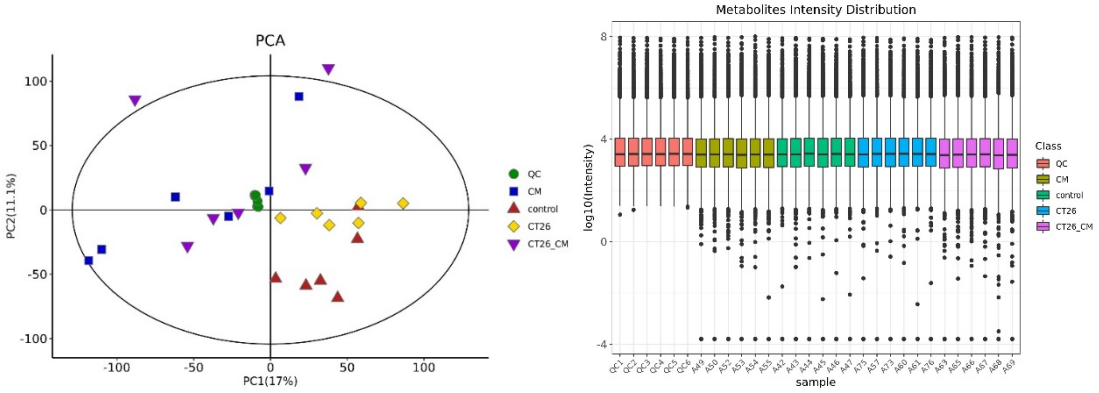


(a) (b)

Figure4 (a) Taxonomic summary of the gut microbiota at phylum level. (b) The heat map of species composition of the gut microbiota in CT26 group and PBS group at genus level.

3.2 The present of tumor affected the absorption of FFCT

A high dose of FFCT (2.6mg/g) was used both in CT26 group (to be CT26-CM group) and PBS group (to be CM group) to compare the serum metabolism between the two groups. In the PCA plots (Figure 5(a)), the quality control (QC) samples were closely gathered in the center of the scoring chat, showing the good stability of the instrument detection in this experiment. In the Metabolites Intensity Distribution box plot (Figure 5(b)), the median line of each group was on a horizontal line, indicating that the samples were relatively stable. And the dots below represented the degree of dispersion, showing a relatively low dispersion in CM group.



(a) (b)

Figure 5 Quality control chart. (a) PCA score plot of all samples. (b) Metabolites Intensity

Distribution box plot of all samples.

The OPLS-DA plot can reflect the variability between groups and within groups, and can observe the general distribution trend among samples. As Figure 6 showed, samples of the two groups were separated clearly, which meant that the repeatability within each group was good, the difference between the two groups was large and the data characteristics between different groups were significantly different.

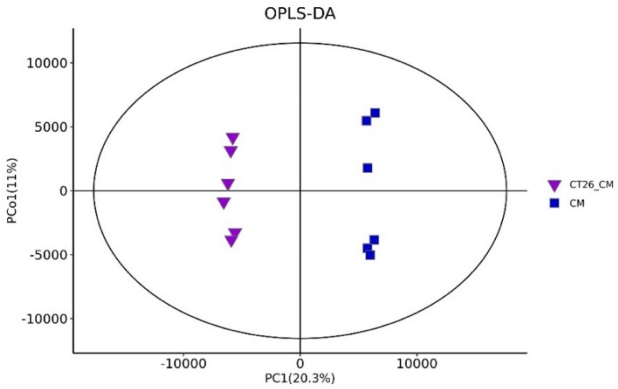
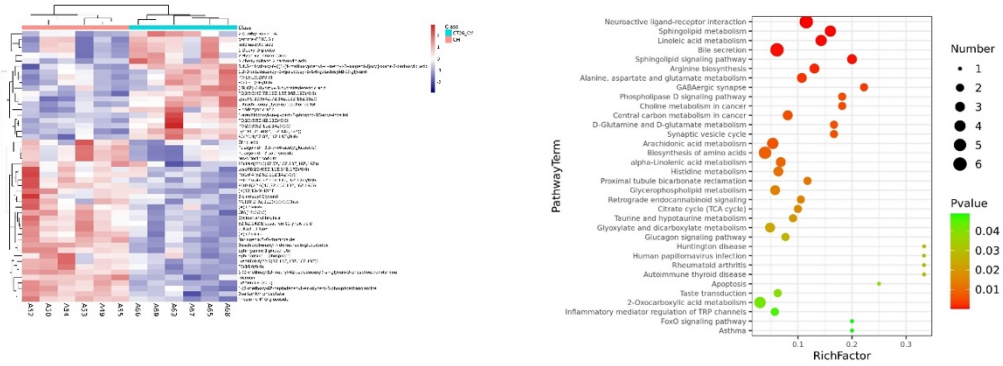


Figure 6 OPLS-DA score plot of tumor-bearing group treating by FFCT (CT26-CM group) and FFCT-treating group (CM group). R2=(0.0,0.981), Q2=(0.0,-0.159).

There were 225 different metabolites between CT26-CM group and CM group. In order to show the relationship between the two groups and the expression differences in metabolites among different samples more intuitively, the hierarchical clustering heatmap was used to visualize the different potential biomarkers between these two groups. Top 50 difference metabolites and KEGG pathways enriched by significantly different metabolites were identified as listed in Figure 7. Combined with network retrieval, serum metabolites that related to FFCT like Citric acid, （±）12-HEPE, Cycloartanyl ferulate, 5Z,8Z,14Z－Eicosatrien－11－ynoic acid , (±)12－HETE, PE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PE(16:0/0:0), LysoPE(18:0/0:0), 1−(2−methoxy−13−methyl−6Z−tetradecenyl)−sn−glycero−3−phosphoethanolamine , Oseltamivir phosphate and 1−(2−methoxy−6Z−heptadecenyl)−sn−glycero−3−phosphoethanolamine were much more in CM group than in CT26-CM group, indicating that the present of tumor could affect the metabolism of FFCT(Figure 7(a)). As Figure 7(b) showed, these differential metabolites of FFCT involved in multiple pathways including the Alanine, aspartate and glutamate metabolism, Central carbon metabolism in cancer, Biosynthesis of amino acids, et al.

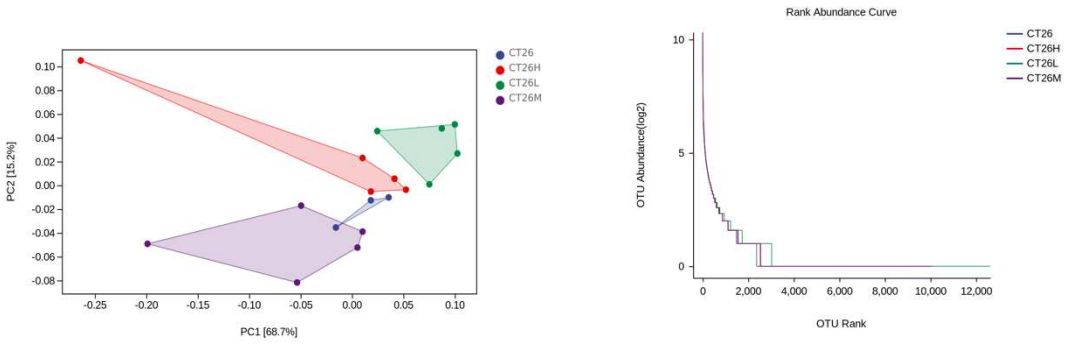


(a) (b)

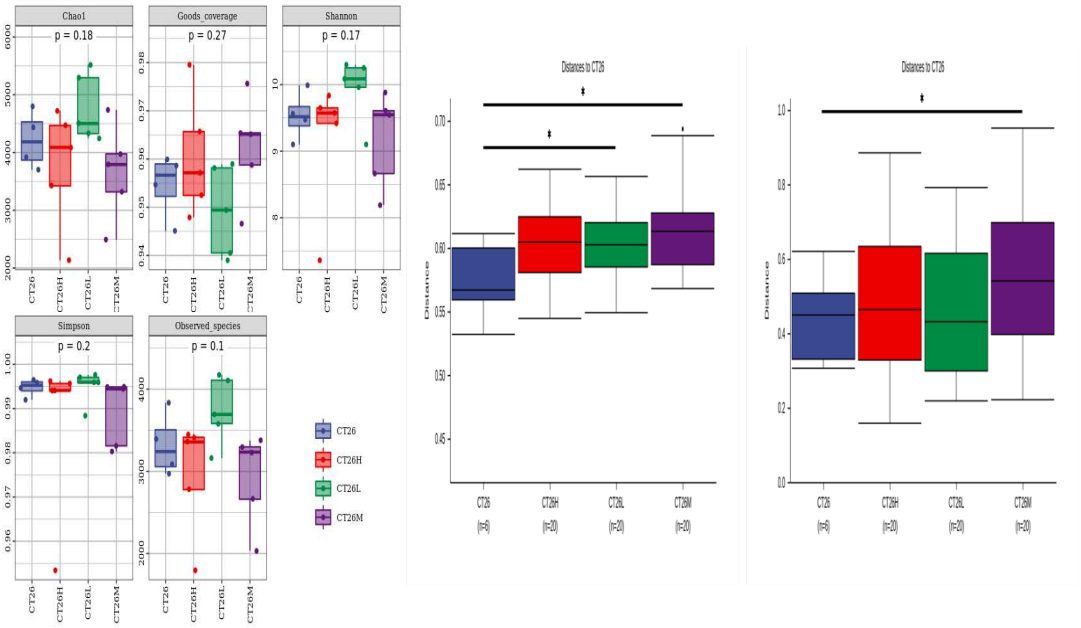
Figure 7 The hierarchical clustering heatmaps. (a) The hierarchical clustering heatmap of different metabolites in CT26-CM group and CM group. (b) KEGG pathways enriched by significantly different metabolites between CT26-CM group and CM group (p<0.05).

3.3 FFCT ameliorated the GM in tumor-bearing mice

As the above results shown, GM and blood metabolism of FFCT between tumor-bearing group and healthy group were quite different. The dysbiosis of GM in tumor-bearing mice was corresponding to less serum metabolites that related to FFCT. Next, 16S rRNA gene sequencing was used to detect the effect of different doses of FFCT on the GM of tumor-bearing mice. As expected, compared with the CRC tumor-bearing group (CT26), FFCT-treating groups (CT26-L, CT26-M, CT26-H) had different composition of GM. The PCA diagram (Figure 8(a)) showed that the species abundance composition of FFCT-treating groups was different from that in CT26 group, and could be classified obviously. The abundance grade curve (Figure 8(b)) showed that the FFCT-treating groups had higher community evenness than CT26 group. The alpha diversity analysis (Figure 8(c)) showed that there were no significant differences in Chao1, Good's coverage, Shannon, Simpson and Observed species indices between CT26 group and FFCT-treating group. However, beta diversity analysis using both unweighted and weighted UniFrac distances indicated that the medium dose FFCT-treating group had higher dissimilarities among gut microbial communities than CT26 group (Table 3,4).



(a) (b)

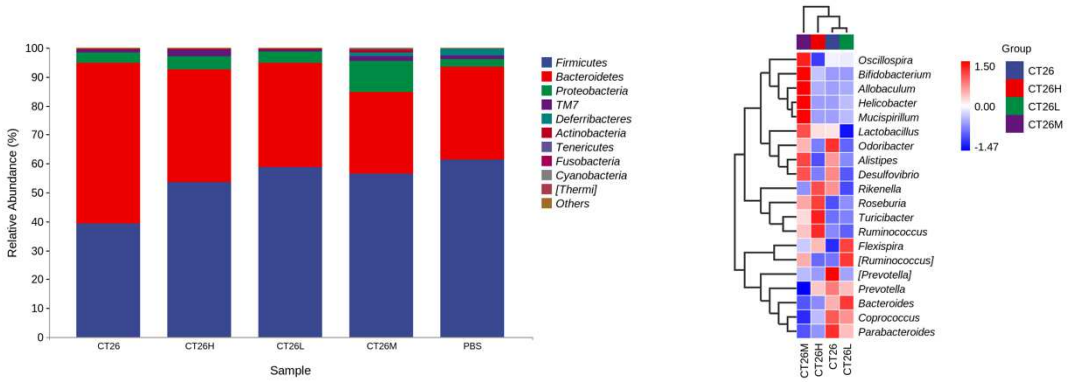


(c) (d) (e)

Figure 8 (a) The PCA diagram of CT26 group and FFCT-treating groups. (b) The abundance grade curve of CT26 group and FFCT-treating groups. (c) The differences in Chao1, Good's coverage, Shannon, Simpson and Observed species indices between CT26 group and FFCT-treating groups. (d) The differences in unweighted UniFrac distance between CT26 group and FFCT-treating groups. (e) The differences in weighted UniFrac distance between CT26 group and FFCT-treating groups.

The taxonomic summary figure (Figure9(a)) showed that the composition of GM in the four groups was mainly characterized by the *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *TM7* and *Deferribacteres*. In the distribution of phyla, FFCT-treating groups had higher abundance of *Firmicutes* than tumor-bearing group, which showed a similar trend with Healthy group. And with the increase in FFCT concentration, the abundance of *Firmicutes* got decreased. On the contrary, FFCT-treating groups had lower abundance of *Bacteroidetes* than tumor-bearing group, which also showed a similar trend with Healthy group. The CT26-M group had the least abundance of *Bacteroidetes* and the most abundance of *Proteobacteria* and *Actinobacteria*.

As showed in Figure9(b), species with high expression in CT26 group but low expression in FFCT-treating groups were as follows: *Odoribacter*, *[Prevotella]*, *Prevotella*, *Coprococcus*, *Parabacteroides*. On the contrary, species like *Roseburia*, *Turicibacter* and *Flexispira* in CT26 group were much less than in FFCT-treating groups. Among these species, *Roseburia* and *Turicibacter* increased with the increase of FFCT concentration.



(a) (b)

Figure9 (a) Taxonomic summary of the gut microbiota at phylum level. (b) The heat map of species composition of the gut microbiota in CT26 group and FFCT-treating groups at genus level.

4 Discussion

In the adjuvant treatment of tumor, Chinese medicine has become an essential part due to its multi-target effect and low toxicity. However, the mechanism of the curative effect is complicated. Studies showed that emodin could induce the cancer cell apoptosis via endoplasmic reticulum stress-dependent events, which produced reactive oxygen species (ROS) and regulated the signaling pathways[23]. Qinrui Han[24] et al., showed that bufarenogin induced intrinsic apoptosis through the cooperation of Bax and adenine-nucleotide translocator. Xiao-Qin Zhu[25] et al., treated CRC HCT-116 xenograft mouse model with Qingjie Fuzheng Granules and found that this compound suppressed tumor cell proliferation through inhibiting SHh pathway. Yang Li [26]et al., did research on Gegen Qinlian decoction. They tested the immune cells, the cytokines and the intestinal mucosa tight protein. Combining with the results of gut microbiota (GM), they concluded that Gegen Qinlian decoction could enhance immunity and protect intestinal barrier function by decreasing the relative abundance of *Megamonas* and *Veillonella*, and increasing the relative abundance of *Bacteroides*, *Akkermansia* and *Prevotella*. As Yang's study shown, GM had become a considerable factor in the development and treatment of CRC.

Jiyoung Ahn[27] et al., tested the community of GM in a study of 47 CRC subjects and 94 control subjects. From such a clinical research, they found that CRC case subjects decreased overall microbial community diversity, showing the potentially modifiable nature of GM and the difference of GM between CRC patients and healthy people. GM is an important part of intestinal mucosal barrier and intestinal immunity. GM can influence physiological and pathological processes throughout the whole body, including the bioavailability and meta-ballism of

macronutrients and micronutrients as well as metabolites. The vast majority of Chinese medicine are absorbed through intestinal tract to achieve the therapeutic effect, so there is the hypothesis that GM could affect the absorption and metabolism of Chinese medicine.

In this study, we chose *Fufangchangtai* (FFCT) formula as the model prescription. FFCT decoction was created by the famous Chinese Medicine Practitioner Professor Jiege Huo through summing up decades of experience in the CRC therapy. The previous multicenter, randomized, double-blind clinical trial had confirmed that FFCT had a certain effect on improving the life quality, enhancing immune function and prolonging the median survival time of CRC patients. However, the mechanism of its anti-tumor effect is unclear, that's the reason for choosing FFCT as the research object.

Instead of AOM/DSS induction mice, the subcutaneous transplantation mice were used as the CRC tumor-bearing mice model. The AOM/DSS induction mice model is induced by administration through gastrointestinal tract, which not only affects the experimental results of GM but also has the disadvantages of long modeling cycle, unstable effect and high mortality. The subcutaneous transplantation mice model is more intuitive and makes the experimental results stable and reliable.

In order to verify the difference in GM among different groups of mice, the GM in fecal of CRC mice and healthy mice was detected. The results showed that the abundance of *Firmicutes* decreased and *Bacteroidetes* increased in tumor-bearing mice, which were consistent with findings from clinical analysis of CRC patients[28]. As our study shown, *Coprococcus*, *Helicobacter*, *Desulfovibrio*, et al., had high expression in CT26 group. *Coprococcus*, which is related to butyrate production[29] and the metabolism of SCFAs[30], increased in tumor-bearing mice. Sarah Vascellari's study proposed that the high expression of *Coprococcus* was associated with the development of gut inflammatory environment and gastrointestinal dysfunctions[31], which were pathological factors of CRC. *Helicobacter*, especially *helicobacter_hepaticus*, can lead to an increase in oxidative phosphorylation that may increase DNA-damaging free radicals[32]. Verena Friedrich[33] colonized the spontaneous fatal colitis transgenic mouse modelled with *helicobacter_hepaticus* and found the disease developed rapidly, showing that this kind of bacteria was a disease driver in the model of colitis and was not conducive to maintain intestinal homeostasis. *Desulfovibrio* is considered as the virulent bacteria which plays a role in destroying colonic mucosa and inducing intestinal inflammation and bacterial translocation[34]. Since the significant difference in GM between CRC tumor-bearing mice (CT26 group) and healthy mice (PBS group), the further study was allowed to be carried out in the tumor-bearing model mice.

We detected the blood metabolism of tumor-bearing mice and healthy mice after

administration of FFCT to explore whether the dysbiosis of GM under the burden of tumor had an impact on serum metabolism that related to TCM. Metabolites that related to FFCT like Citric acid, (±) 12-HEPE, Cycloartanyl ferulate, PE(16:0/0:0), et al., were much more in CM group than in CT26-CM group. Citric acid is the product of tricarboxylic acid cycle, helping transform food into usable energy. Studies[35-37] showed that citric acid could enhance the nutrient absorption including enhancing bioavailability of minerals and the absorption of zinc supplements. Moreover, citric was identified to have high correlation coefficients with proangiogenic activity[38]. (±) 12-HEPE is a kind of circulating hydroxy polyunsaturated fatty acids[39], and is recently identified as BAT (brown adipose tissue) –derived circulating factors (BATokines), which plays a critical important role in regulating the systemic lipid pool that related to energy dissipation[40]. Cycloartenyl ferulate is a component of gamma-oryzanol, which was found to have the function of capturing IgE and attenuating the allergic reaction[41]. Islam et al., investigated the effect of γ-oryzanol, Cycloartenyl ferulate and ferulic acid on a model of colitis in mice. The research found that Cycloartenyl ferulate had strong antioxidant effects so to inhibit NF-κB activity and then to ameliorate colonic inflammation[42], showing the potential for treating or preventing the gastrointestinal inflammatory diseases. The above three main different metabolites indicated that FFCT produced a marked effect on providing nutrition and energy and regulating microenvironment. The results suggested that there were more serum metabolites that related to FFCT in healthy group. Combing with the results of GM, the conclusion could be summarized that a relatively healthy intestinal flora and microenvironment was conductive to the absorption and metabolism of drugs into the blood, so as to play a better therapeutic effect.

Further analysis of metabolic pathways showed that the therapeutic effect of FFCT might be predominantly relevant to the ability of regulating Alanine, aspartate and glutamate metabolism, Central carbon metabolism in cancer, Biosynthesis of amino acids. Alanine, aspartate and glutamate metabolism involves in the synthesis of arginine and purine, the interconversion of C3 and C4, and the activity of the DcuS-DcuR two-component system[43]. Ryosei Sakai et al., investigated permeability and metabolism of dietary glutamate in Caco-2 intestinal epithelial cell layer model and found that the intestinal epithelial cell monolayer could utilize dietary glutamate to maintain glutamate homeostasis in the body[44]. Central carbon metabolism traditionally includes glycolysis pathway (EMP), pentose phosphate pathway (PPP) and tricarboxylic acid cycle (TCA), and it is the main source of energy required by organisms and provides precursors to other metabolism in the body. As amino acids are the basic units of protein molecules and give play to activating immune system in organisms such as immunoglobulin synthesis, lymphocyte quantity and phagocyte function[45]. These results indicated that FFCT could keep the balance of

internal environment through intestinal absorption and metabolism so as to involve in the synthesis of substance and supplement of energy in serum metabolism.

At the same time, FFCT was administrated to CRC tumor-bearing mice to observe the alteration of GM under the intervention of TCM. It was also found that there were significant differences in the composition of GM in tumor-bearing mice before and after FFCT administration. Many evidences demonstrated that *Firmicutes* was a phylum with documented anti-tumorigenic effects[46]. *Bacteroidetes* on the contrary, could drive DNA damages in colon epithelial cells as a potential "driver" of CRC[47]. As the abundance of *Firmicutes* was higher and the abundance of *Bacteroidetes* was lower in FFCT-treating groups, and *Firmicutes*/*Bacteroidetes* was generally regarded as significant relevance in signaling GM status[48], the results indicated that the imbalance of GM in tumor-bearing mice was corrected. The intervention of FFCT increased the abundance of *Turicibacter* and *Roseburia*, and as the FFCT concentration increased, the abundance of these two species of phylum increased. *Turicibacter* is involved in fermentation metabolism and its main metabolite is lactic acid, which has the function of regulating muscle and anti fatigue. *Roseburia* can ferment a variety of carbohydrates, increase the content of butyric acid in the intestine, and has the effect of preventing or treating obesity related diseases. Studies showed that *Turicibacter* and *Roseburia* had a negative association with intestinal dystrophy[49]. Combining with the research results, the conclusion could be drawn that FFCT changed the structure of GM and maintained the enteral nutrition. However, the specific intestinal protection mechanism remains to be further studied.

5 Conclusion

It was pointed out that the GM should be concerned during the therapy of FFCT. The more healthier intestinal microenvironment was conductive to the better efficacy of FFCT. On the contrary, a dysbiosis GM under CRC burden blocked the absorption and metabolism of FFCT. In addition, FFCT could correct the imbalance GM of CRC individual. This research provided a new viewpoint to study the therapeutic effect of traditional Chinese medicine from the perspective of combining gut microbiota and serum metabolism, and also provided a basis for improving the GM status of CRC patients in clinical treatment, aiming to achieve better curative effect.

Declarations

Funding

This research was funded by Jiangsu Postgraduate Research and Practice Innovation Program (Grant Number SJCX20_0519): Research on anti colorectal cancer mechanism of Fufangchangtai based on gut microbiota.

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Contributions

Mengmeng Cai performed laboratory work and wrote first draft. Zhibing Lin developed the concept, managed samples collection, and supervised laboratory work and data analysis. Ya Xiao performed revision and contributed to writing manuscript. Jinmiao Lu, Xiaoyu Wang, Shilan Zhu and Xiaoyu Chen performed laboratory work. Jialin Gu and Yuzhu Ma managed samples collection. Jiege Huo and Zhaoguo Chen supervised, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

The study was approved by the Laboratory animal welfare and Ethics Management Committee of Shanghai Veterinary Research Institute (ethics number: SHVRI-SZ-20200420-01, SHVRI-SZ-20200720-01).

Consent for publication

All the authors have read the paper before submission and consent submuission.

Competing interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Acknowledgements

For the completion of my thesis, first, I wish to express my deepest gratitude to my supervisor, Prof. Jiege Huo and Prof. Zhaoguo chen, who has given me the most valuable suggestions and advice, and made necessary corrections. Then I am greatly grateful to Dr. Zhibing Lin and Dr. Jinmiao Lu for their help in my laboratory work and data analysis.

I am also deeply indebted to all the other teachers and colleagues for their direct and indirect help to me.

Special thanks should go to my parents and friends for their continuous support and encouragement.

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Figures

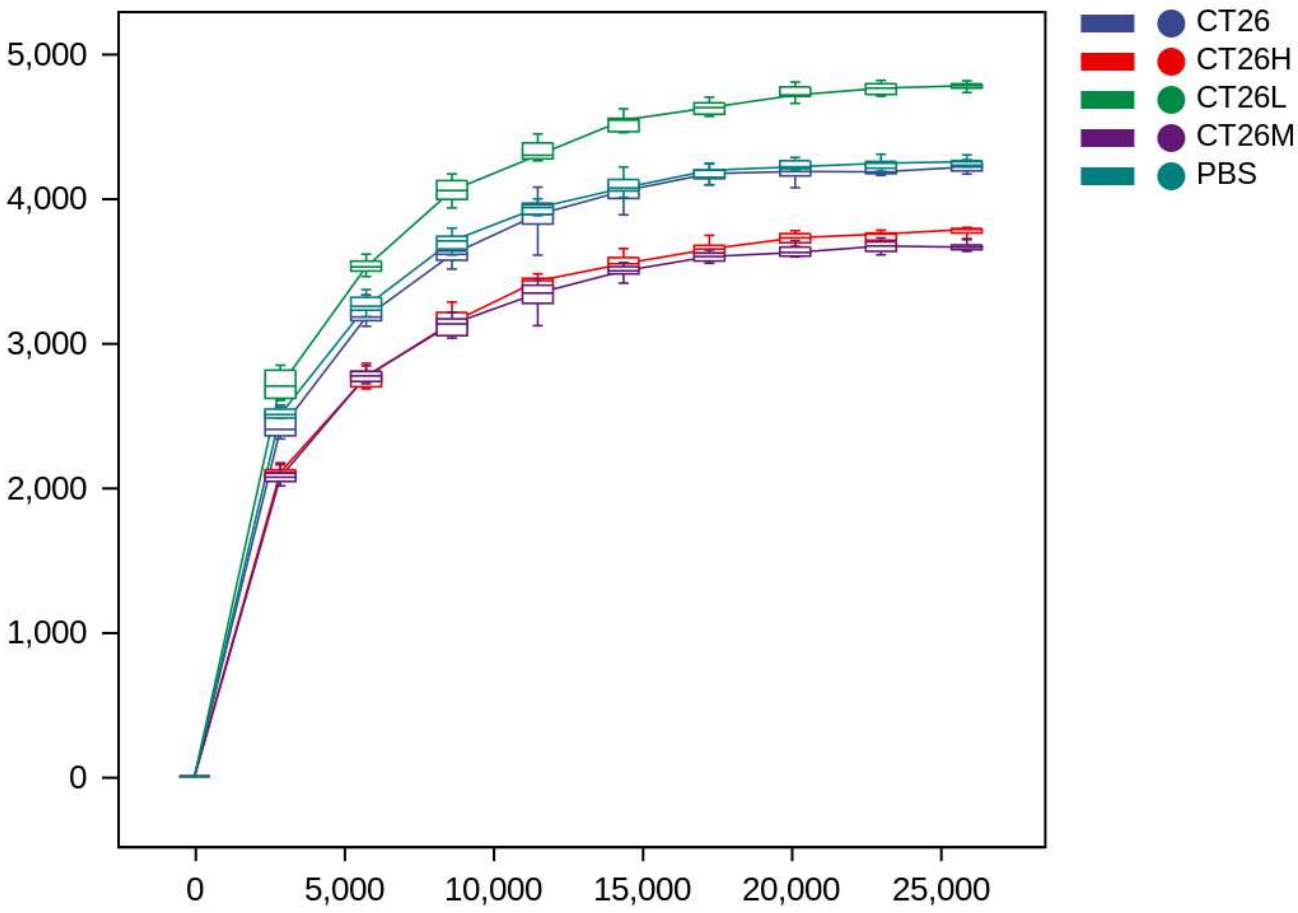

Figure 1

The rarefaction curves of each group based on Chao1

Figure 2

Venn diagram of OTUs in each group

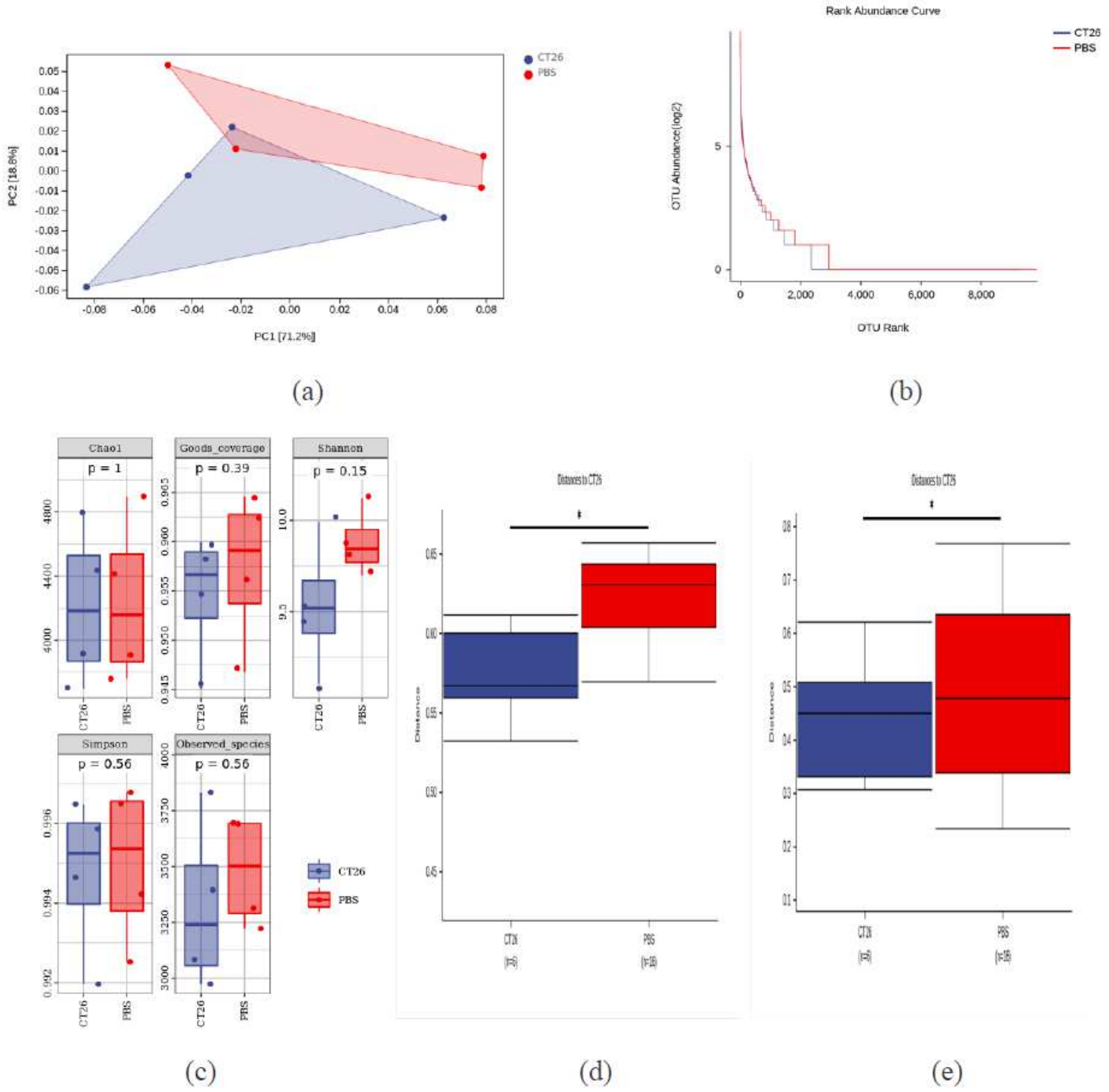

Figure 3

(a) The PCA diagram of CT26 group and PBS group. (b) The abundance grade curve of CT26 group and PBS group. (c) The differences in Chao1, Good's coverage, Shannon, Simpson and Observed species indices between CT26 group and PBS group. (d) The differences in unweighted UniFrac distance between CT26 group and PBS group. (e) The differences in weighted UniFrac distance between CT26 group and PBS group.

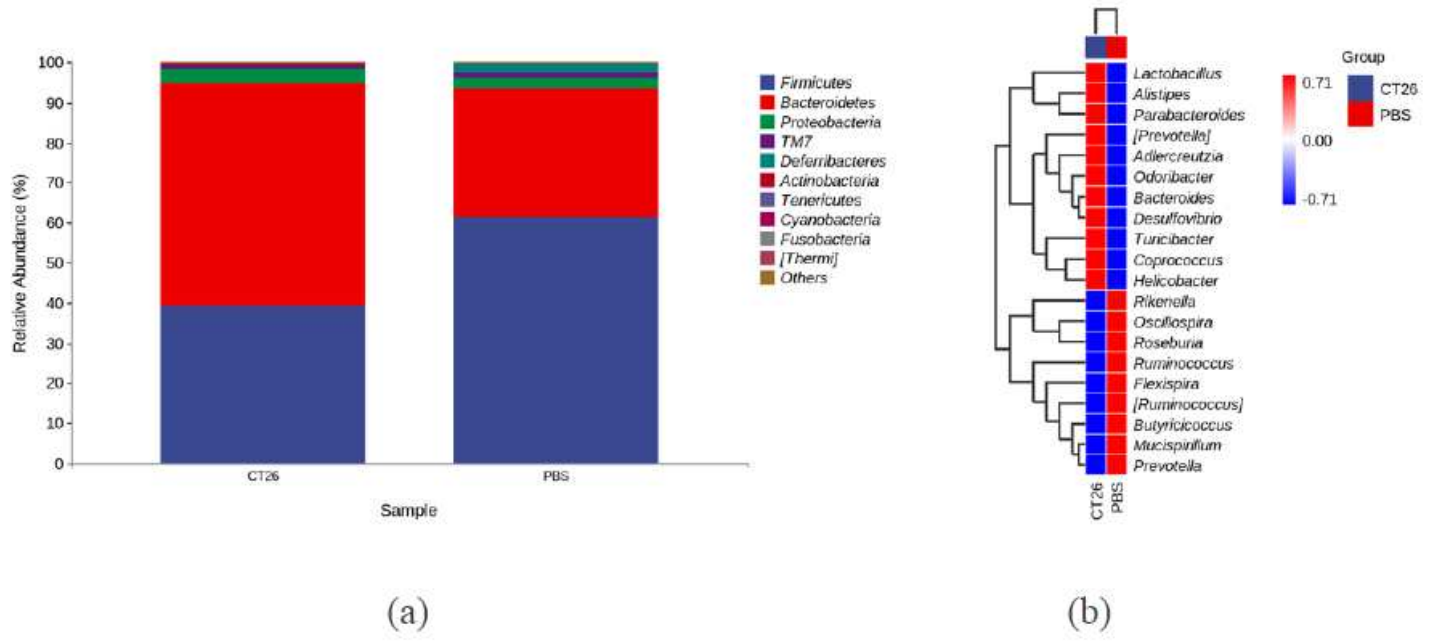

Figure 4

(a) Taxonomic summary of the gut microbiota at phylum level. (b) The heat map of species composition of the gut microbiota in CT26 group and PBS group at genus level.

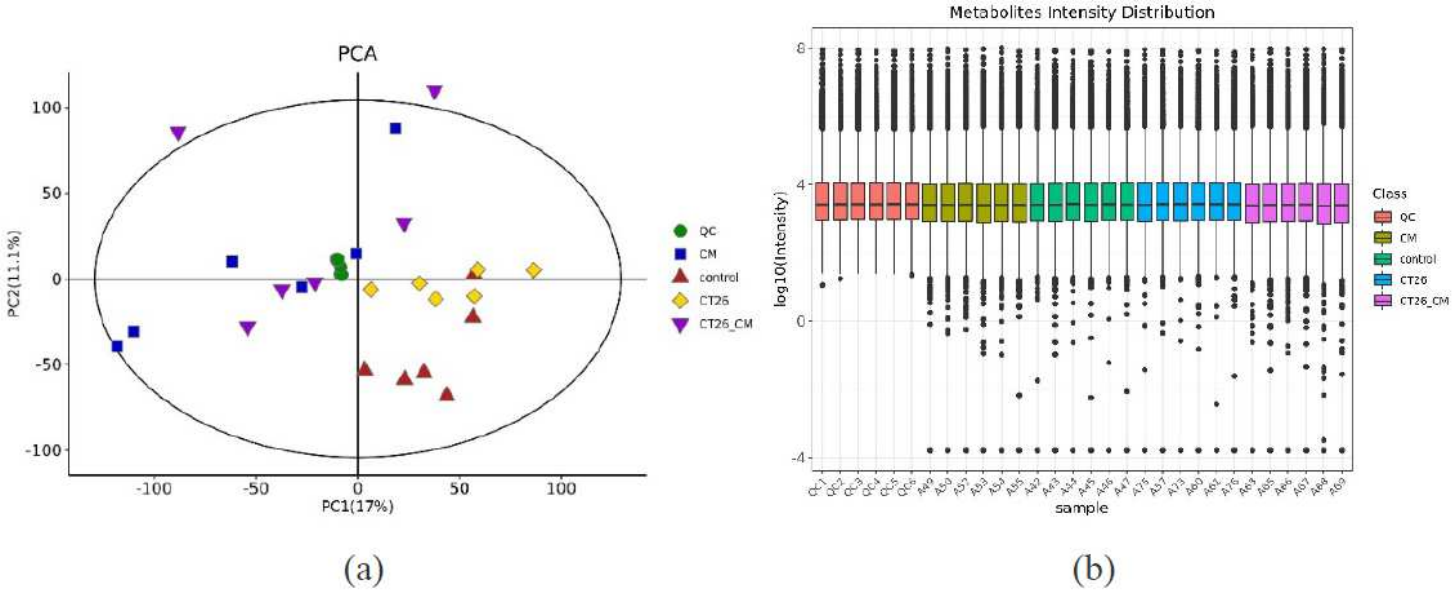

Figure 5

Quality control chart. (a) PCA score plot of all samples. (b) Metabolites Intensity Distribution box plot of all samples.

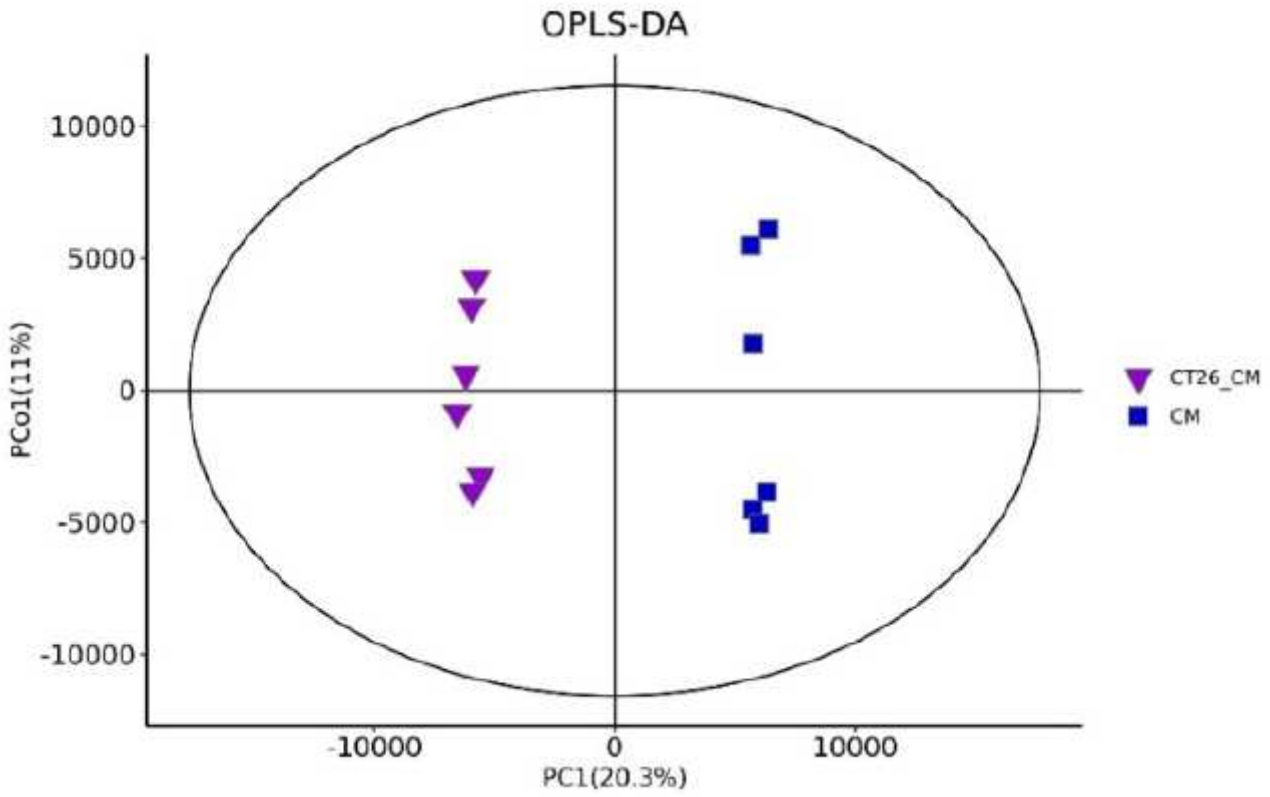

Figure 6

OPLS-DA score plot of tumor-bearing group treating by FFCT (CT26-CM group) and FFCT-treating group (CM group). R2=(0.0,0.981), Q2=(0.0,-0.159).

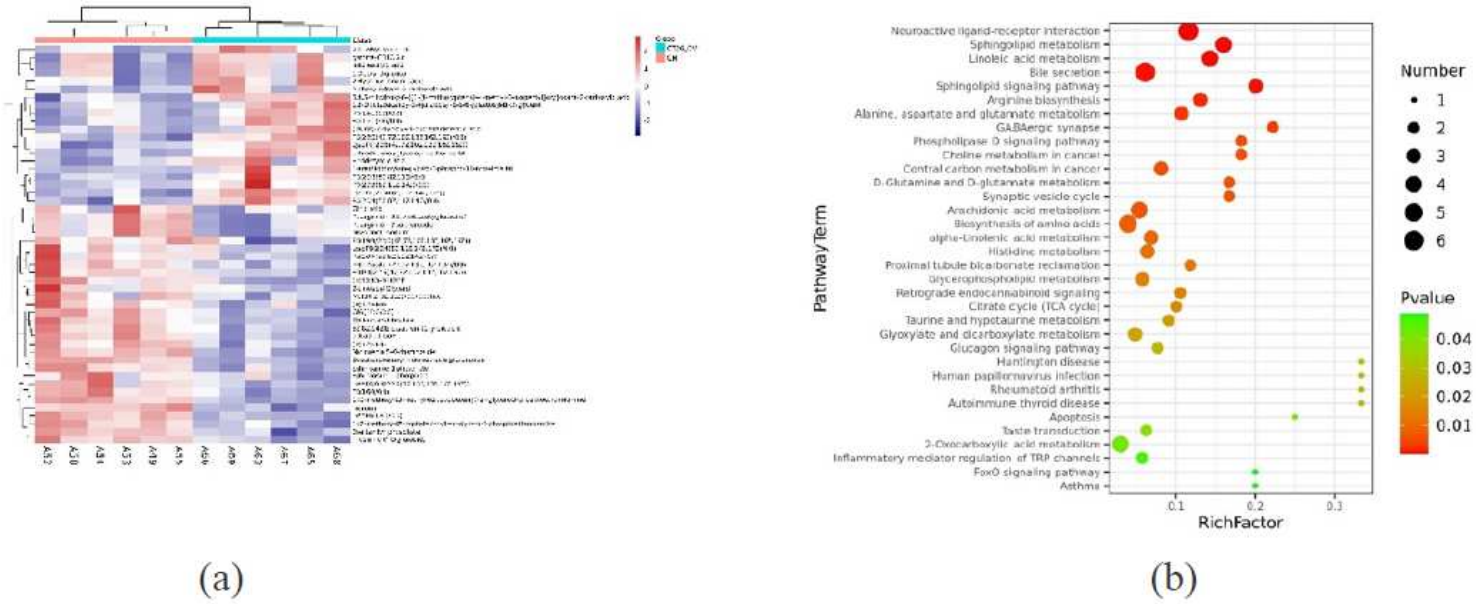

Figure 7

The hierarchical clustering heatmaps. (a) The hierarchical clustering heatmap of different metabolites in CT26-CM group and CM group. (b) KEGG pathways enriched by significantly different metabolites

between CT26-CM group and CM group (p<0.05).

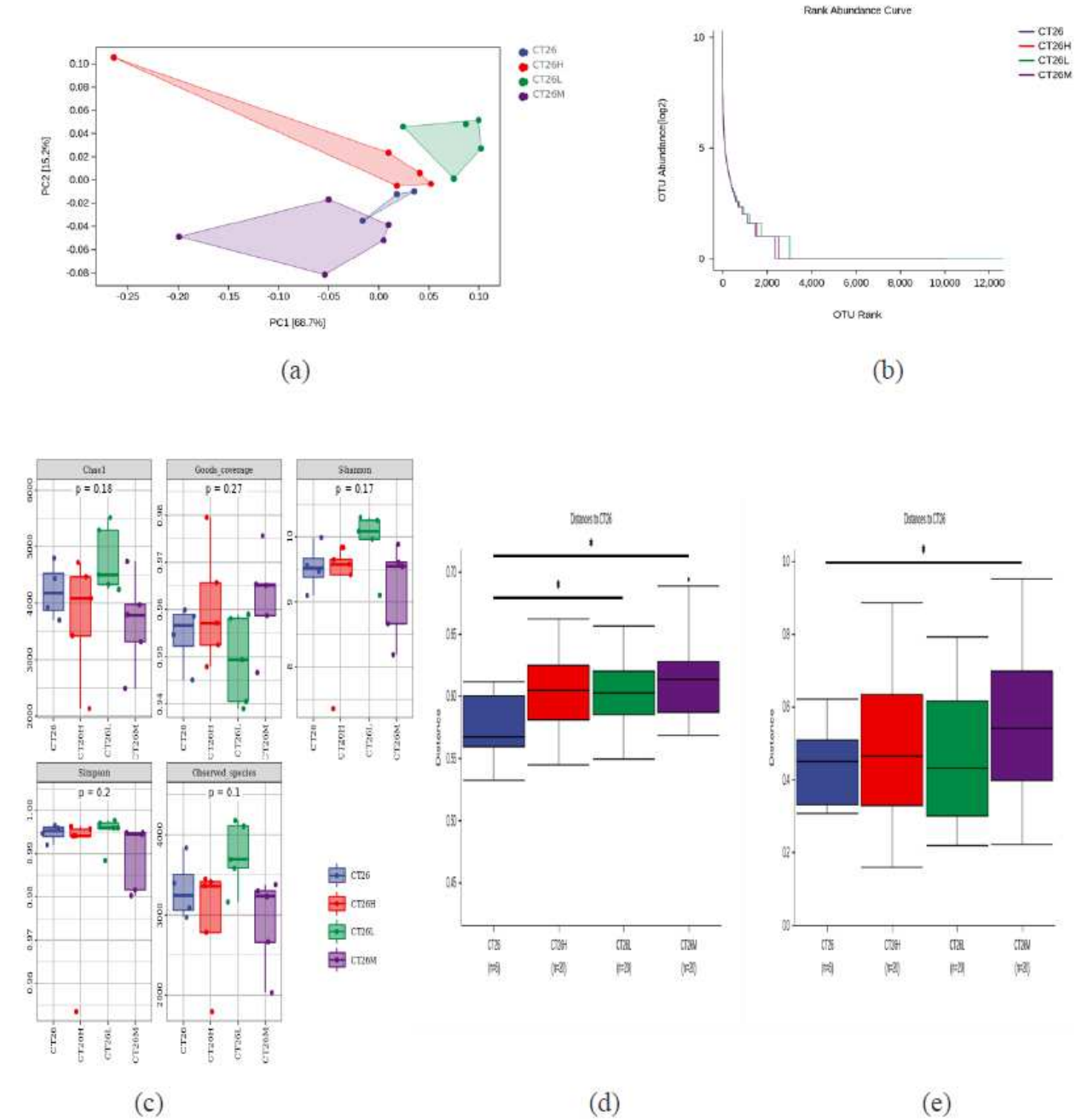

Figure 8

(a) The PCA diagram of CT26 group and FFCT-treating groups. (b) The abundance grade curve of CT26 group and FFCT-treating groups. (c) The differences in Chao1, Good's coverage, Shannon, Simpson and Observed species indices between CT26 group and FFCT-treating groups. (d) The differences in

unweighted UniFrac distance between CT26 group and FFCT-treating groups. (e) The differences in weighted UniFrac distance between CT26 group and FFCT-treating groups.

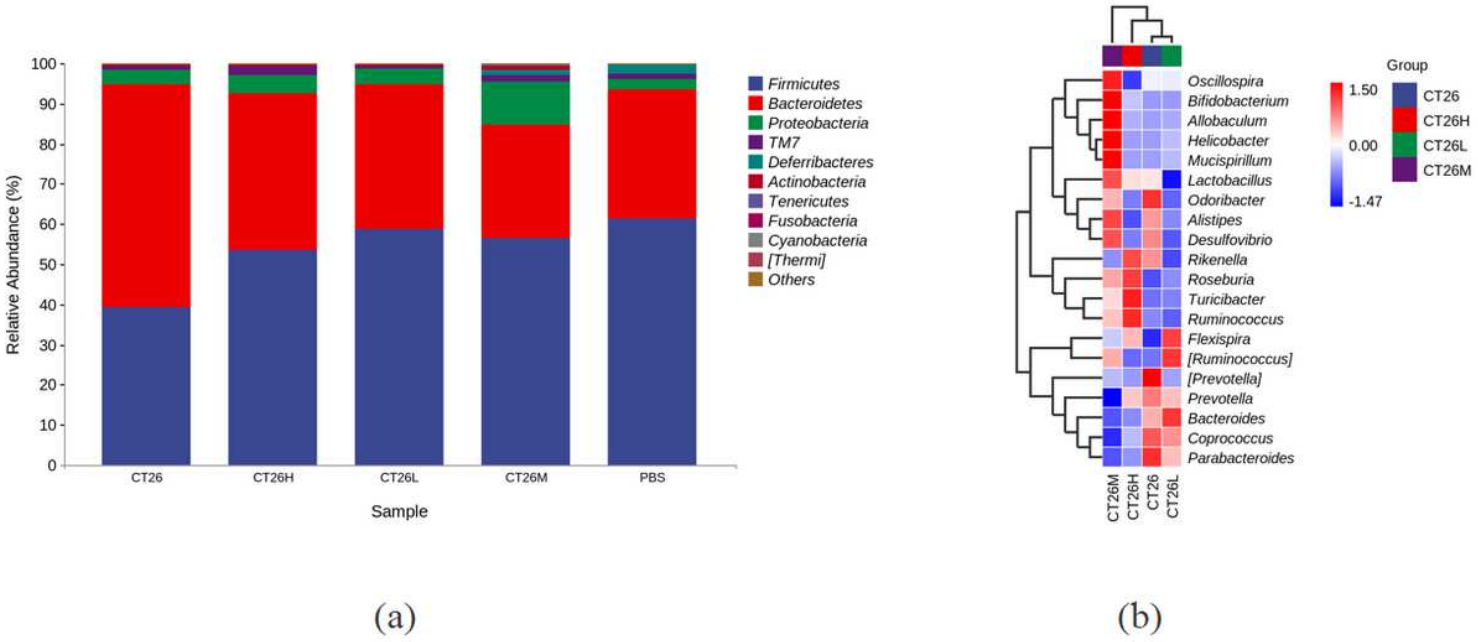

Figure 9

(a) Taxonomic summary of the gut microbiota at phylum level. (b) The heat map of species composition of the gut microbiota in CT26 group and FFCT-treating groups at genus level.

Supplementary Files

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- T1unweightUnifracCT26PBS.xls.xlsx
- T2weightUnifracCT26PBS.xls.xlsx
- T3unweightUnifracCT26CM.xls.xlsx
- T4weightUnifracCT26CM.xls.xlsx