



Associations between gut microbiota and thyroidal function status in Chinese patients with Graves' disease

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Abstract

Objective The imbalance of gut microbiota has been linked to manifold endocrine diseases, but the association with Graves' disease (GD) is still unclear. The purpose of this study was to investigate the correlation between human gut microbiota and clinical characteristics and thyroidal functional status of GD.

Methods 14 healthy volunteers (CG) and 15 patients with primary GD (HG) were recruited as subjects. 16SrDNA high-throughput sequencing was performed on IlluminaMiSeq platform to analyze the characteristics of gut microbiota in patients with GD. Among them, the thyroid function of 13 patients basically recovered after treatment with anti-thyroid drugs (oral administration of Methimazole for 3–5 months). The fecal samples of patients after treatment (TG) were sequenced again, to further explore and investigate the potential relationship between dysbacteriosis and GD.

Results In terms of alpha diversity index, the observed OTUs, Simpson and Shannon indices of gut microbiota in patients with GD were significantly lower than those in healthy volunteers ($P < 0.05$). The difference of bacteria species was mainly reflected in the genus level, in which the relative abundance of *Lactobacillus*, *Veillonella* and *Streptococcus* increased significantly in GD. After the improvement of thyroid function, a significant reduction at the genus level were *Blautia*, *Corynebacter*, *Ruminococcus* and *Streptococcus*, while *Phascolarctobacterium* increased significantly ($P < 0.05$). According to Spearman correlation analysis, the correlation between the level of thyrotropin receptor antibody (TRAb) and the relative abundance of *Lactobacillus* and *Ruminococcus* was positive, while *Synergistetes* and *Phascolarctobacterium* showed a negative correlation with TRAb. Besides, there were highly significant negative correlation between *Synergistetes* and clinical variables of TRAb, TPOAb and TGAbs ($P < 0.05$, $R < -0.6$).

Conclusions This study revealed that functional status and TRAb level in GD were associated with composition and biological function in the gut microbiota, with *Synergistetes* and *Phascolarctobacterium* protecting the thyroid probably, while *Ruminococcus* and *Lactobacillus* may be novel biomarkers of GD.

Keywords Gut microbiota · Graves' disease · TRAb · *Ruminococcus* · *Lactobacillus*

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Introduction

A complex microbial ecosystem in the intestines of human hosts is constituted by gut microflora, which is of great significance to human health [1, 2]. The gut microbiota plays vital roles in immunomodulation, metabolism, barrier protection and nutrition absorption in humans [3, 4]. Numerous

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studies have shown a close correlation between the alteration of gut microbiota and metabolic diseases such as obesity or diabetes, and the two may interact as both cause and effect. The dysbiosis of intestinal microflora is considered to chiefly produce an effect on the glycolipid metabolism in the host recently. There are significant quantitative and qualitative differences between the gut microbiota of the lean and fat people, as well as between the diabetic and the healthy subjects [5–8]. There is a consensus that obesity is usually associated with a decrease in Bacteroides and an increase in Firmicutes [9–13], but this conclusion is currently controversial [14–16]. Undeniably, gut microbiota and its metabolites can participate in host nutritional absorption and metabolism by regulating appetite, fat production, gluconeogenesis and inflammation [17]. Therefore, gut microbiota is probably one of the contributing factors of metabolic diseases such as obesity and diabetes. Further analysis of gut microbiota and its metabolites is crucial.

At present, related studies on gut microbiota and thyroid disease have been widely reported at home and abroad. Among them, there are still inconsistent conclusions about GD and gut microbiota. Hyperthyroidism refers to the hyperactivity of thyroid and the release of redundant-free thyroid hormone, which leads to hypermetabolism throughout the body [18]. GD is the most common form of hyperthyroidism. A considerable portion of hyperthyroidism is caused by GD, an organ-specific autoimmune disease characterized by thyrotoxicosis, thyroncus and the production of TRAb. TRAb stimulates the proliferation and hypersecretion of thyroid cells, leading to autoimmune hyperthyroidism [19]. Zhou and his team found that the number of Bifidobacterium and Lactobacillus decreased significantly while Enterococcus increased in patients with hyperthyroidism [20]. The study of Ishaq showed the obvious increase of Haemophilus parainfluenza in patients with GD, which may be one of the candidate pathogens [21]. Shi reported an evident raise in the abundance of Bacteroidetes in patients with Graves' ophthalmopathy [22]. Although these studies have found differences in the gut microbiota between hyperthyroid patients and normal persons in terms of composition and diversity, however, there may be insufficient understanding of the role of gut microbiota in the occurrence and development of GD due to the individual differences of subjects, the inadequateness of sample size and the depth of sequencing. Besides, there are few studies on the relevance between gut microbiota and TRAb levels.

In this study, the 16S rDNA sequencing of fecal samples comes from 15 patients with primary GD and 14 healthy volunteers were performed and the thyroid-related antibodies of their blood were detected. Moreover, the treatment group was set up, aiming to find out the remarkable feature of gut microbiota of patients with GD that had never located. The association between the detected characteristic flora and the

serum thyroid antibodies of GD patients was analyzed, so as to put forward the new insights for the pathogenesis of GD.

Methods

Sample selection

15 patients with primary GD were recruited as the hyperthyroidism group (HG) from February 2018 to April 2019 in the Department of Endocrinology, Jinling Hospital of Southeast University, School of Medical. In the meantime, 14 healthy volunteers were recruited as the control group (CG). All the patients with GD were only treated with anti-thyroid drugs (Methimazole, Merck Company). And then, specimens were taken again after 3–5 months of treatment when subjects' thyroid function is basically restored to normal, a total of 13 cases were taken as the treatment group (TG). All patients with GD met the diagnostic criteria of the "Guideline for the Diagnosis and Treatment of Chinese Thyroid Diseases", and the enrolled cases without treatment were excluded from transient hyperthyroidism such as subacute thyroiditis, painless thyroiditis and drug-induced hyperthyroidism. All the subjects were defined between 18 and 65 years old. None of the subjects had history of autoimmune disease or long-term hormone treatment, diabetes and other metabolic diseases, constipation or chronic diarrhea such as inflammatory bowel disease (IBD), cancer and familial genetic disease, severe dysfunction of multiple organ such as heart, liver and kidney. No one had taken antibiotics or microbiological preparations within one month prior to participation. We also excluded vegetarians and pregnant or lactating women. And the remained subjects were without history of gastrointestinal surgery and addiction of alcohol or drug. Besides, the patients and the controls were all from the local area of Jiangsu Province, located in the east coast of China, and were on a similar diet. Prior to the collection of clinical information and samples, all subjects signed an informed consent, meeting the requirements of ethical review.

Sample collection

The stool specimens were collected by the subjects themselves, and the procedure of collection was as follows: discharge the stool on a piece of dry and clean paper, pick up the center part with the pick rod of the sterile stool sample box and put into the stool sample box immediately. After that, the researcher placed the specimen on ice instantly to make sure that the temperature was lower than 4 °C and transported to the endocrinology laboratory within one hour. Then the central part was picked out in the ventilation cupboard after disinfection and sterilization within 30 min. All of the samples were divided into two parts, about the size of

soybean grains, and stored in 1.5-mL EP tubes. The samples were frozen at -80°C refrigerator promptly after collection. The collection and storage of samples were conducted in strict accordance with the rules and regulations of the Department of Endocrinology, Jinling Hospital of Southeast University, School of Medical, and all samples had no history of repeated freeze–thaw and contamination.

DNA extraction and PCR amplification

DNA extraction and concentration determination are carried out according to the instructions of Magen Hipure Soil DNA kit and Qubit @dsDNA HS Assay kit. Then the 16 s rDNA V3-V4 variable region was amplified by PCR with upstream primer 5'-CCTACGRRBGCASCAGKVRV-GAAT-3' and downstream primer 5'-GGACTACNVGGG TWTCTAATCC-3'. The amplification procedure is as follows: pre-denaturation at 94°C for 3 min, denaturation at 94°C for 5 s, annealing at 57°C for 90 s, extension at 72°C for 10 s, additional extension at 72°C for 5 min, and there are altogether 24 cycles. The 25 μL amplification system is composed of 2.5 μL TransStart buffer, 2 μL dNTPs, two of 1 μL primer, 0.5 μL TransStart polymerase, 20 ng DNA template and the rest supplemented with ddH₂O. The specificity and size of PCR products were detected by 1.5% agarose gel electrophoresis. The amplified PCR products were purified and eluted, then the library was constructed which was detected and quantified by Qubit3.0 Fluorometer fluorescence quantitative instrument (Invitrogen, Carlsbad, CA), and finally analyzed on the computer.

16S rDNA gene sequencing and data processing

Perform the PE250/PE300 double-terminal sequencing according to the instructions of the Illumina MiSeq sequencer, read the sequencing data by the MiSeq Control Software (MCS) of MiSeq, perform quality control on the original sequencing data, and obtain valid data after assembly and filtering. Amplicon sequences with 99% similarity were classified as an operational taxonomy unit (OTU), then QIIME software was used for OUT clustering and species annotation, and species abundance and diversity index analysis were performed based on the results of OTU cluster analysis. The statistical analysis of the composition structure of gut microbiota was carried out at various taxonomic levels such as phylum level and genus level.

Statistical analysis

Histogram of colony composition and heat map of species distribution among groups were drawn by *R*-language. The abundance of a species in a group was the average of all sample abundances of the species under the group. Obtain

significant differences between groups with Kruskal–Wallis non-parametric test ($q < 0.05$), linear discriminant analysis (LDA) and LDA effect size (LEfSe) (LDAscore > 2.0), draw the heatmap of the distribution of different species based on *R*-language. In addition, Spearman rank test was used to analyze the correlation between gut microbiota and thyroid-related antibodies.

Results

Baseline characteristics

There was no obvious difference in age, blood pressure and body mass index between 15 patients with GD (7 males and 8 females) and 14 healthy volunteers (6 males and 8 females) (Table 1). The relevant clinical variables between group HG and CG were compared through the independent samples *t* test in SPSS (Table 1). The related indexes between group HG and TG (6 males and 7 females) were analyzed by paired-samples *t* test. As shown in Table 2, the thyroid function indexes such as FT3 and FT4 in group TG have basically recovered and TRAb decreased significantly. The data obtained were expressed as mean \pm standard deviation ($x \pm s$), with $P < 0.05$ representing statistically significant differences.

Alpha diversity

Alpha diversity index was used to evaluate the species abundance and diversity of microflora [23], in which Goods Coverage could reflect the depth of sample detection, Simpson index and Shannon index were a reflection of microflora diversity, Chao1 index and observation-OTUs index were a reflex of microflora richness. The Goods coverage of each group was more than 99.9%, indicating that the sequencing results of gut microbiota represent the true situation of the sample. The sample dilution curve of this study was drawn according to observed-OTUs. As shown in Fig. 1a and b, the curve has been smooth when the amount of sequencing data reached more than 5000, showing that the amount of sequencing data was sufficient to cover all species in the samples. Therefore, the sequencing data of this study was extremely reasonable. Compared with the CG group, the abundance and diversity of gut microbiota in group HG decreased significantly, among which the Shannon, Simpson and observation-OTUs indexes were visibly declined (Table 1, $P < 0.05$). After improvement of thyroid function, the Shannon index and Chao1 index in the TG group increased significantly (Table 2, $P < 0.05$). This was also confirmed by the Venn diagram of OTU distribution (Fig. 1c, d), which intuitively showed the common and unique number of OTU in each group. In addition to

Table 1 Index of the CG group and HG group

Index	CG (<i>n</i> = 14)	HG (<i>n</i> = 15)	<i>P</i>
Baseline characteristics			
Age	27.29 ± 5.73	28.87 ± 6.79	0.505
BMI	22.30 ± 3.38	20.86 ± 1.95	0.166
SBP	115.79 ± 13.39	122.75 ± 10.47	0.221
DBP	74.93 ± 8.71	81.13 ± 11.38	0.166
HR	73.71 ± 10.54	89.08 ± 16.41	0.008
ALT	16.64 ± 7.26	28.5 ± 11.64	0.003
AST	17.14 ± 3.06	23.29 ± 8.31	0.015
UA	317.71 ± 79.45	355.1 ± 56.24	0.216
BUN	4.70 ± 1.29	4.61 ± 1.35	0.866
TG	0.80 ± 0.32	0.95 ± 0.37	0.317
TC	4.37 ± 0.69	3.70 ± 0.96	0.066
Glu	4.86 ± 0.37	5.13 ± 0.24	0.066
Clinical variables			
TSH	1.90 ± 0.63	0.05 ± 0.13	0.000*
T3	1.40 ± 0.33	4.64 ± 2.20	0.000*
T4	107.06 ± 24.23	275.56 ± 66.20	0.000*
FT3	5.05 ± 0.61	22.18 ± 10.44	0.000*
FT4	11.51 ± 1.16	51.45 ± 18.63	0.000*
TGAB	10.78 ± 1.70	983.65 ± 1717.64	0.075
TPOAB	8.63 ± 6.63	110.55 ± 118.50	0.010*
TRAB	0.30 ± 0.00	6.62 ± 5.39	0.001*
Alpha diversity index (<i>x</i> ± <i>s</i>)			
Observed-otus	211.43 ± 68.63	155.80 ± 67.82	0.037*
Chao1	245.68 ± 68.02	202.74 ± 59.55	0.081
Shannon	5.19 ± 0.55	4.55 ± 0.74	0.014*
Simpson	0.95 ± 0.02	0.89 ± 0.07	0.009*
Relative abundance of gut microbiota			
Firmicutes	0.738	0.680	0.267
Bacteroidetes	0.194	0.226	0.506
F/B	3.79	3.01	0.444
Synergistetes	0.0024	0.000012	0.028*
Veillonella	0.0006	0.0172	0.039*
KW test between the HG group and the CG group at genus level (<i>P</i> / <i>Q</i>)			
Lactobacillus	0.0005	0.0000	0.0006/0.0039
Veillonella	0.0029	0.0003	0.0446/0.0446
Phascolarctobacterium	0.0015	0.0063	0.0324/0.0370
Coprococcus	0.0021	0.0090	0.0259/0.0346
Anaerostipes	0.0008	0.0061	0.0144/0.0230
Turicibacter	0.0000	0.0002	0.0010/0.0039
Cloacibacillus	0.0000	0.0000	0.0066/0.0152
Mogibacterium	0.0000	0.0000	0.0076/0.0152

The data obtained are expressed as mean ± standard deviation (*x* ± *s*), with *P* < 0.05 representing statistically significant differences. The two groups were analyzed with independent t-test. Reference range (T3: 1.01–2.48 nmol/l, T4: 71–161 nmol/l, FT3: 3.8–6.50 pmol/l, FT4: 7.9–17.2 pmol/l, TSH: 0.49–4.5mIU/l, TRAB: 0.00–1.5 IU/l, TGAB: 0.2–95 IU/ml, TPOAB: < 30 IU/ml). The missing rate of each variable was less than 20%. The abundance of a species in a group is the average of the abundance of all samples of the species in the group

Table 2 Index of the HG group and TG group ($x \pm s$)

Index	HG($n=13$)	TG($n=13$)	P值
Clinical variables			
TSH	0.06 ± 0.14	2.53 ± 5.79	0.152
T3	4.69 ± 2.31	1.72 ± 0.59	0.002*
T4	294.76 ± 60.61	119.07 ± 38.81	0.000*
FT3	23.08 ± 10.65	5.44 ± 1.48	0.000*
FT4	52.70 ± 17.67	11.46 ± 5.03	0.000*
TRAb	5.91 ± 4.62	2.08 ± 1.64	0.004*
TPOAB	80.76 ± 102.4	149.57 ± 193.02	0.460
TGAB	857.85 ± 1756.85	182.07 ± 232.72	0.380
Alpha diversity index ($x \pm s$)			
observed-otus	159.38 ± 50.94	191.62 ± 36.10	0.002*
chao1	191.97 ± 51.58	224.37 ± 37.98	0.016*
shannon	4.46 ± 0.74	4.56 ± 0.48	0.535
simpson	0.89 ± 0.07	0.91 ± 0.03	0.327
Relative abundance of GUT microbiota			
Streptococcus	0.0231	0.0021	0.021*
Blautia	0.0425	0.0142	0.013*
Ruminococcus	0.0234	0.0122	0.015*
Veillonella	0.0196	0.0029	0.066
Phascolarctobacterium	0.0031	0.0184	0.021*
KW test between the HG group and the TG group at genus level(P/Q)			
Blautia	0.0066	0.0337	0.0001/0.0010
Streptococcus	0.0009	0.0158	0.0012/0.0040
Phascolarctobacterium	0.0124	0.0018	0.0030/0.0069
Ruminococcus	0.0077	0.0194	0.0322/0.0375
Lachnospira	0.0074	0.0034	0.0404/0.0404
Lactococcus	0.0000	0.0000	0.0076/0.0133
Corynebacterium	0.0000	0.0000	0.0107/0.0150

The two groups were analyzed with paired-samples t test

the common parts, the controls had the most unique OTUs, while primary GD patients had a significant reduction, and the OTUs was significantly improved after treatment.

Species composition of gut microbiota

The composition and structure of gut microbiota between GD patients and healthy volunteers were significantly different at the phylum and genus levels. From the phylum level, the histogram (Fig. 2a, b) showed that the dominant bacteria of both patients and controls were *Firmicutes* and *Bacteroidetes*, and the ratio of the two (F/B) was 3.79 in CG and 3.01 in HG (Table 1, $P=0.444$). The results were not statistically significant probably because of individual differences or small sample size in the research. As can be seen from the heatmap (Fig. 3a, c), the relative abundances of *Proteobacteria* and *Synergistetes* in HG were significantly reduced compared with the CG group, while the *Proteobacteria* in TG was significantly increased compared with the HG group. From the genus level (Figs. 2b, d, 3c, d), there

was a high relative abundance of *Phascolarctobacterium* in the CG group. Compared with CG, the relative abundances of *Lactobacillus*, *Veillonella* and *Streptococcus* increased in HG. Statistical analysis showed that *Veillonella* (Table 1, $P=0.039$) had significant difference. Compared with HG, the abundance of *Phascolarctobacterium* increased, while *Blautia*, *Lactobacillus*, *Veillonella* and *Streptococcus* decreased in the TG group. Statistical analysis showed that the differences were statistically significant except for *Veillonella* (Table 2, $P < 0.05$).

Differential species analysis

Kruskal–Wallis test is used to get the P value based on the species abundance table. And the Q value was obtained after the P value was modified by FDR (Benjamini and Hochberg False Discovery Rate). With $Q < 0.05$ as the threshold value, the species with significant difference between groups were obtained. Among them, no significant differences in species among groups were obtained at the phylum level of

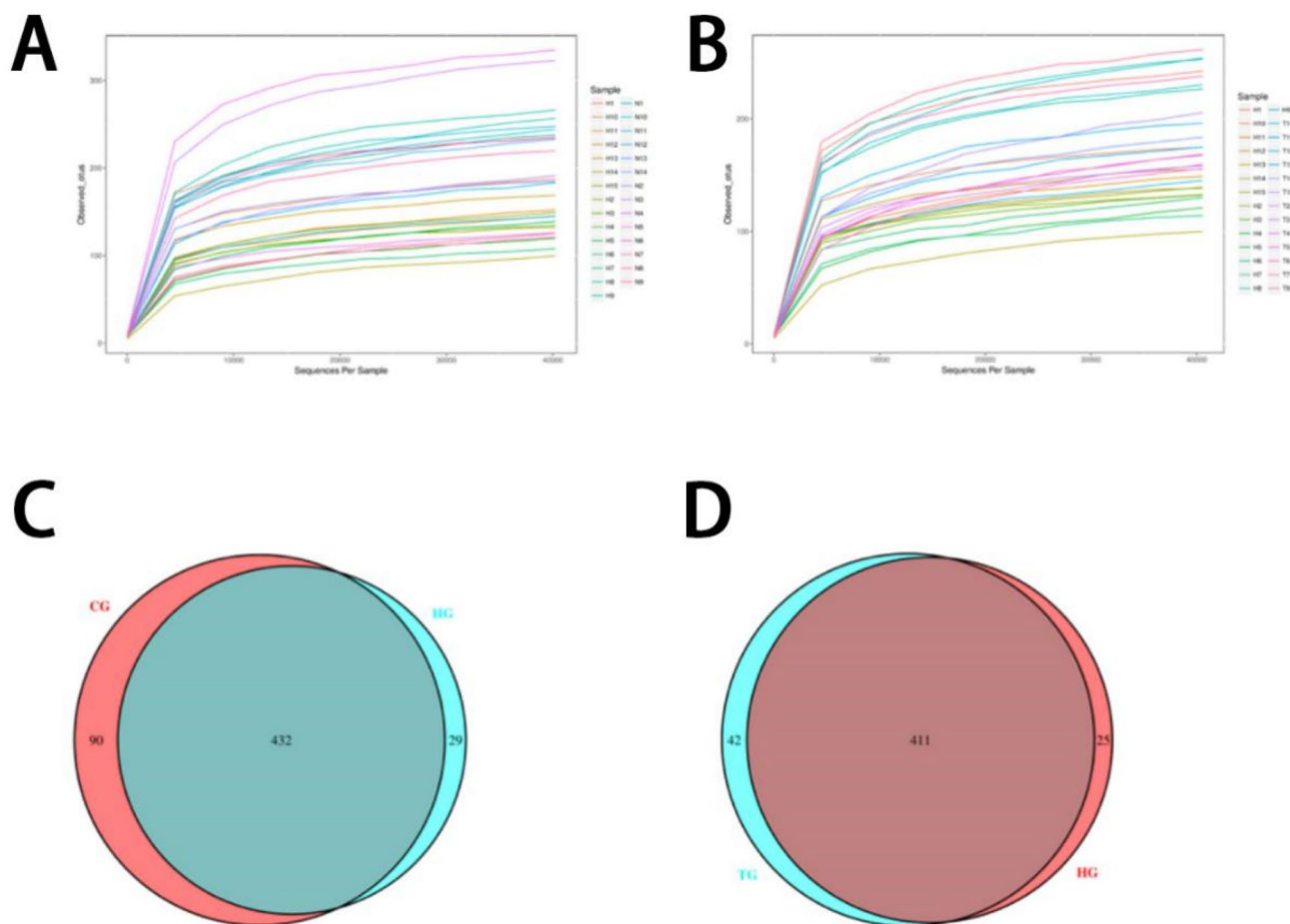


Fig. 1 Dilution curve (a, b) and Venn diagram (c, d)

each group. The analysis of different species at genus level (Tables 1, 2) and LDA analysis (Fig. 4) showed that, compared with the CG group, *Phascolarctobacterium* decreased significantly in HG, while the relative abundances of microorganisms were significantly increased, including *Streptococcus*, *Blautia*, *Lactobacillus*, *Veillonella*, *Bacilli* and so on. Compared with HG, the relative abundances of *Ruminococcus*, *Streptococcus* and *Blautia* decreased significantly, while the abundance of *Phascolarctobacterium* increased significantly in TG.

Potential correlation between gut microbiota and thyroid-related antibodies

Spearman correlation analysis was used to explore the potential correlation between different bacterial abundances and clinical thyroid-related antibodies in GD patients (Fig. 4c). We calculated the Spearman correlation coefficient R between gut microbiota and immune indexes including TRAb. As shown in Table 3, among all, *Synergistetes* was negatively correlated with TRAb ($P=0.000$,

$R = -0.702$), TGAAb ($P=0.003$, $R = -0.624$) and TPOAb ($P=0.001$, $R = -0.711$), while *Lactobacillus* was positively correlated with TRAb ($P=0.024$, $R=0.489$) and TPOAb ($P=0.006$, $R=0.607$). TRAb was also negatively correlated with *Phascolarctobacterium* ($P=0.011$, $R = -0.544$). Through the correlation analysis of the indexes and gut microbiota of GD patients before and after treatment (Table 4), it was found that there was a positive correlation between TRAb and *Ruminococcus* ($P=0.021$, $R=0.553$). We also analyzed the correlation between the changes of index and bacterial abundance in GD patients before and after treatment (Fig. 4d, e). The results indicated that the abundance difference value of *Ruminococcus* ($P=0.035$, $R=0.638$) and *Phascolarctobacterium* ($P=0.046$, $R=0.610$) had a highly positive correlation with the difference value of TRAb level. Therefore, it is speculated that the increase of TRAb level in GD patients is related to the increase of *Ruminococcus* and *Lactobacillus*, as well as the decrease of *Phascolarctobacterium* and *Synergistetes*. These microbiota may be involved in the immune process of GD.

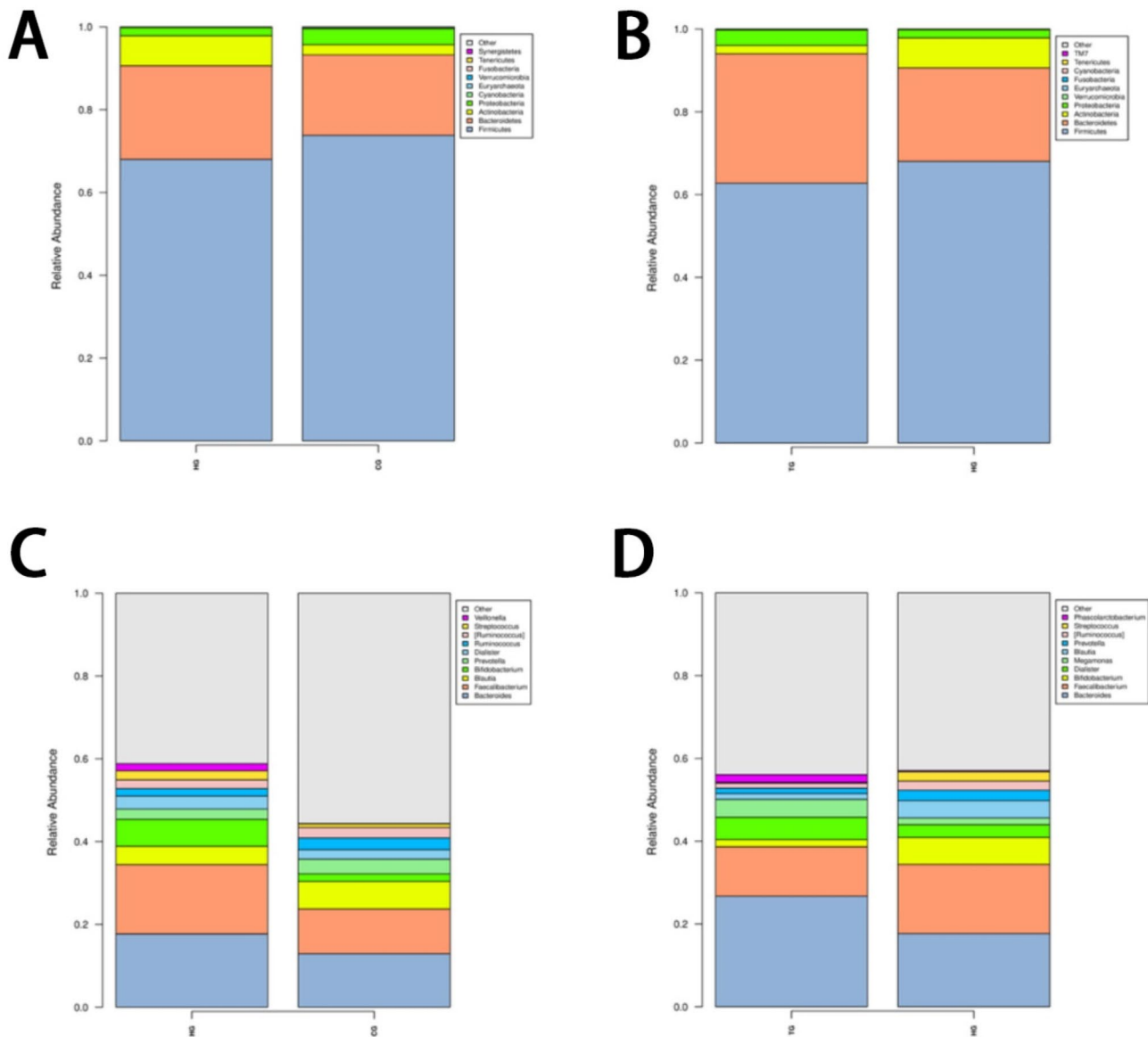


Fig. 2 Histogram of gut microbiota at the phylum (a, b) or genus (c, d) level

Discussion

The gut microbiota plays an important role in gastrointestinal homeostasis and may be one of the contributing factors of thyroid disease [24, 25]. Patients with GD are prone to suffer from digestive system symptoms such as hunger, anorexia, vomiting and increased defecation [26, 27]. The increased intestinal motility in patients may lead to changes in intestinal physiology, and thereby further remodeling gut microbiota composition [28]. Through the detection of gut microbiota and thyroid-related antibodies in 14 normal subjects and 15 GD patients, as well as the analysis of the changes of microflora and serum antibodies in GD patients before and after treatment, this study explored the

correlation between gut microbiota and immune status represented by TRAb in GD, to provide new biological indicators for GD, new insights for the pathogenesis of GD and a new perspective for the treatment of GD. In this study, we found that the gut microbiota of GD patients had significant changes compared with the controls. The abundance and diversity of gut microbiota were significantly reduced in GD patients, while increased apparently after treatment along with the improvement of microflora.

The alpha diversity and richness of gut microbiota in patients with GD were significantly lower than those in healthy volunteers, which was in agreement with some previous findings [29]. In addition, we observed that when the thyroid function of these GD patients basically recovered

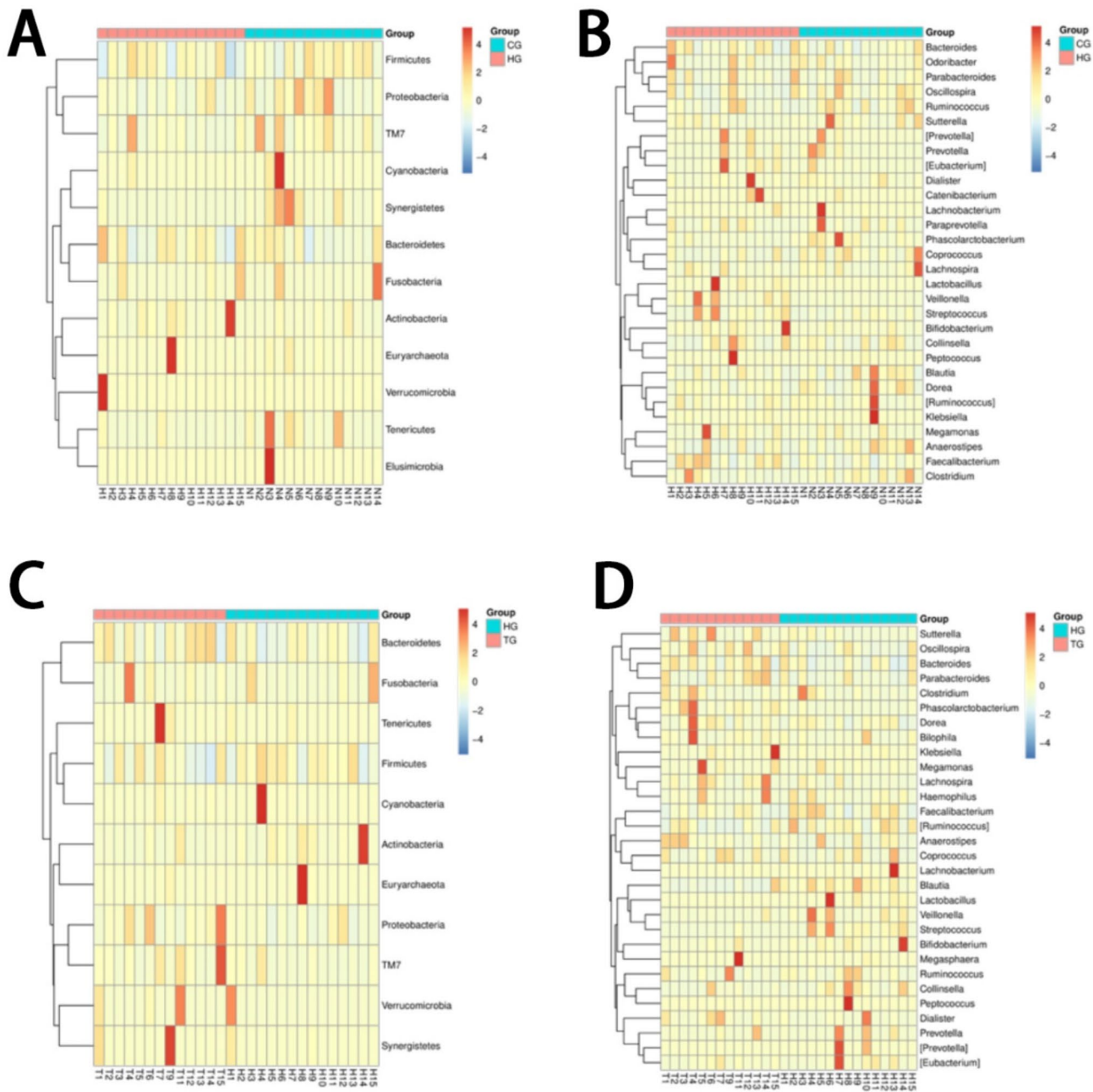


Fig. 3 Heatmap of gut microbiota between each group at the phylum (a, c) or genus (b, d) level

after treatment, the microflora diversity index was also improved. Therefore, we believe that the decrease of bacterial diversity may be closely related to the progress of the disease.

At the phylum level, *Synergistetes* of CG subjects were significantly increased compared to that of HG patients. *Synergistetes* is more common in oral diseases such as periodontitis [29]. Spearman correlation analysis showed that the relative abundance of *Synergistetes* was negatively correlated with TRAb, TGAb and TPOAb levels, suggesting

a possible association between *Synergistetes* and thyroid autoantibodies. A study found that, in SLE patients [30], the abundance of intestinal *Synergetics* was negatively correlated with serum anti-dsDNA antibody titer, and positively correlated with protective natural IgM antibodies against phospholcholine, which indicated that the deficiency of synergetics in SLE patients resulted in compatible synthesis or secret of anti-phospholcholine IgM in B1 cells. Patricia et al. [31] confirmed through in vitro culture that the flora isolated from the feces of SLE patients can promote the activation

of immature CD4+ lymphocytes and the differentiation of Th17 cells, and prevent them from differentiating into Tregs, while Bifidobacterium supplementation can inhibit the over activation of CD4+ lymphocytes. In recent years, other studies [32] have confirmed that some bacteria can affect the differentiation balance of Th17/Treg by secreting inflammatory cytokines or small molecules such as polysaccharide a, short chain fatty acids, etc. These studies suggest that *synergistetes* may be involved in regulating the synthesis and secretion of autoantibodies in patients with autoimmune diseases by affecting the differentiation balance of Th17/Treg. Our study shows that the number of intestinal *Synergistes* in patients with Gd is reduced, which is consistent with the previous studies. Therefore, we speculate that the decrease of *Synergistes* in GD patients may lead to the balance of Th17/Treg differentiation, which further mediates the synthesis and secretion of serum antibodies such as TRAb, TGA and TPOAb.

Ruminococcus and *Lactobacillus* were abundant in the intestines of patients with GD. And we found the two genus were positively associated with the level of TRAb. Compared with HG patients, the relative abundance of *Phascolarctobacterium* in both of the CG and TG subjects was significantly increased. And *Phascolarctobacterium* was negatively correlated with TRAb. *Phascolarctobacterium* colonizes and exerts beneficial effects in the human gastrointestinal tract, producing short-chain fatty acids (SCFAs) including acetate and propionate. A previous study showed that SCFAs and their receptors play an important role in gastrointestinal physiology and pathology, and participate in metabolism and emotion regulation of the host [33]. Moreover, The involvement of SCFAs in the regulation of intestinal motility, hormone secretion, maintain epithelial barrier and immune function, could be of great significance for inflammation and immune response under pathological conditions [34]. The decrease of *Phascolarctobacterium* may lead to the imbalance of immune homeostasis of human host, making it more susceptible to digestive system diseases and metabolic diseases. To some extent, the reduction of the levels of *Phascolarctobacterium* in GD patients may promote the progress and aggravation of the disease.

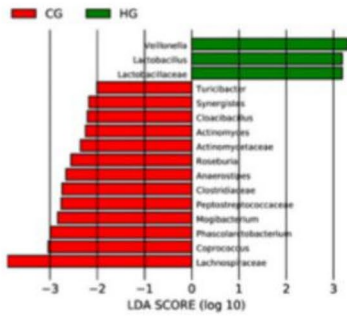
Ruminococcus can produce propionate [35], a kind of short chain fatty acid, which can induce the differentiation of regulatory T cells to produce cytokines such as IL-10 [35]. It has negative effects on intestinal health in inflammatory and allergic diseases [37]. *Lactobacillus* is rarely pathogenic in the human body. Contrarily, it plays an important role in gut microbiota improving and defecation, and has a certain preventive and therapeutic role in inflammatory bowel disease, immune disease and even cancer. The diarrhea symptoms of GD patients may be associated with the high level of *Lactobacillus*. It was found that *Lactobacillus* can induce macrophages to secrete various effector cytokines such as

IL-10, IL-8, IL-6 and TNF- α [38]. And several studies have discovered that *Ruminococcus* and *Lactobacillus* participate in the pathological process of autoimmune diseases through cytokines such as TNF- α , IL-6 and IL-10. Previous studies have found that TNF- α and IL-6 are significantly correlated with Graves' disease [39–42]. TNF- α has been discovered to play an important role in the initiation of humoral immunity, participating in the upregulation of HLA class I, the activation of phagocytes and the induction of IL-1, IL-6 and TNF- α itself, as well as enhancing the expression of HLA II class in cooperation with IFN- γ [43–45], thus inducing cell apoptosis and participating in the regulation of thyroid autoimmunity [46]. However, some studies have found that *Ruminococcus* decrease in certain autoimmune diseases, such as autoimmune hepatitis [47], and induces metabolic diseases. Therefore, the homeostasis of *Ruminococcus* is crucial in autoimmune diseases. Speculate that the increase or decrease of *Ruminococcus* abundance will induce different immune responses. Short chain fatty acids produced by *Ruminococcus* can induce the differentiation of regulatory T cells, increase the number of Th1 and Th17 cells, and upregulate IL-10 which plays key parts in T cell proliferation and B cell differentiation, stimulating B lymphocyte responses and promoting TRAb production by enhancing B cell HLA class II expression and stimulating B cell proliferation [48]. And when *Ruminococcus* abundance is substantially decreased, the activation of effector T cell cause redundant local and systemic inflammatory responses [49], leading to the occurrence of certain autoimmune diseases. Therefore, *Ruminococcus* and *Lactobacillus* were speculated to regulate the secretion of cytokines which leads to the promotion of TRAb production and eventually participate in the immune mechanism of GD.

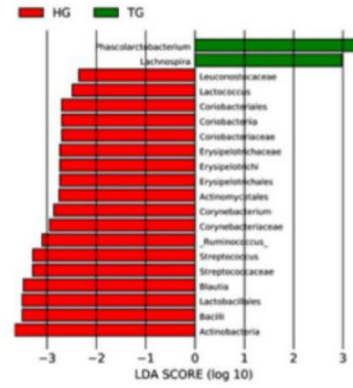
Veillonella is composed of a variety of Gram-negative bacteria and appears in the development of many common oral diseases, such as dental caries and periodontitis [50, 51]. Studies have reported that *Veillonella* acts as a pathogen in a variety of inflammation such as periodontitis, bacteremia and pneumonia [52–54]. It can recognize microorganisms and metabolites through Toll-like receptors as well as trigger inflammatory and immune responses to pathogens [55]. *Streptococcus* is also a pathogenic bacterium of many diseases. It has been found that *Veillonella* and *Streptococcus* coexist in the ecosystem. There is a metabolic interaction between the two [56], which can induce dendritic cells to secrete cytokines, and significantly enhance the response of cytokines such as IL-8, IL-6, IL-10 and TNF- α [57]. Excessive immune response may break the tolerance of thyroid autoimmunity. Thus, we hypothesized that *Veillonella* and *Streptococcus* may play an auxiliary role in the pathogenesis of autoimmune hyperthyroidism under certain circumstances.

Blautia had a significant increase in patients with GD. It can produce butyrate and acetate, and reduce body weight

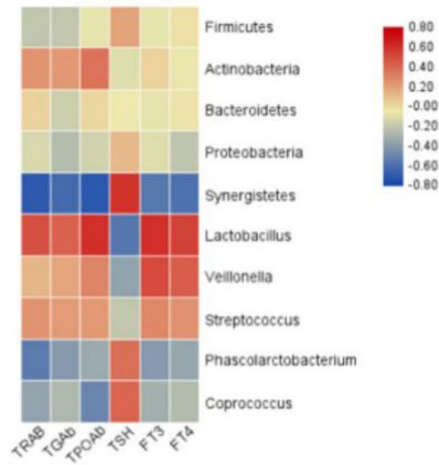
A



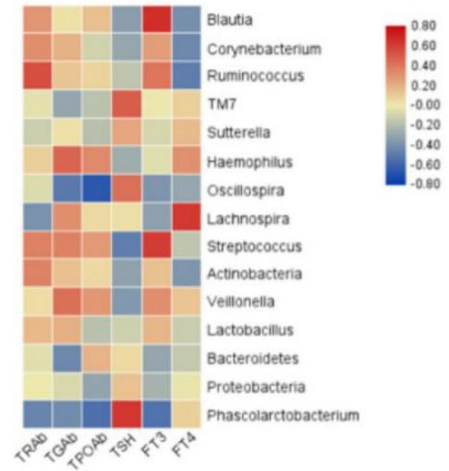
B



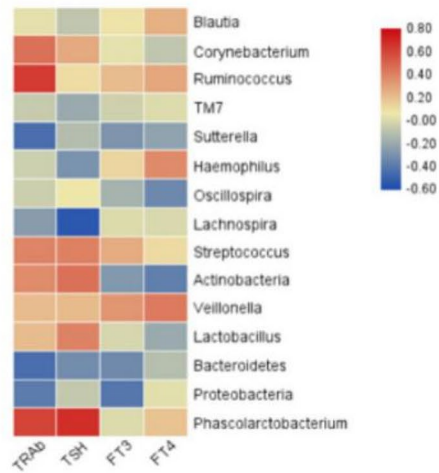
C



D



E



◀**Fig. 4** Histogram based on LDA analysis (a, b), showing the differential flora with LDA score > 2.0. Heatmap based on Spearman correlation analysis between HG and CG (c), HG and TG (d, e, where Δ stands for the difference value)

by regulating G-protein-coupled receptors 41 (GPR41) and GPR43 [58, 59]. In addition, it has been found that *Blautia* is negatively correlated with visceral fat accumulation [60]. The latest research also shows that *Blautia* is consumed in large amounts in the intestinal tract of obese subjects [61]. Therefore, we believe that the emaciation of GD patients may not only be caused by the high metabolism of thyroid hormone. *Blautia* is likely to play a promoting role in the emaciation of GD patients.

In summary, GD has a significant correlation with the composition and structure of gut microbiota, but the microbiota composition found in GD patients might be an effect of hyperthyroidism and might not necessarily be involved in the pathogenesis. The direct role of gut microbiota in the occurrence and development of GD remains to be elucidated in the future research. The main advantages of this study are the collection of samples before the treatment and the setting of the treatment group for the matching analysis of fecal samples before and after the treatment. But there are still some limitations in this study. For one hand, individual differences among samples are inevitable, including incomplete control of drug, diet, environment and other confounding factors. For another, the insufficient sample size and the

limitations of 16SrDNA sequencing method itself are also the defects of the research. The results of the study can only provide evidence of relevance rather than causality. Further research needs to expand the sample size and establish animal models to explore the mechanism of specific microflora in depth.

Conclusion

In brief, the analysis of bacterial community diversity showed that the gut microbiota diversity of GD patients was lower than that in healthy controls and subjects with thyroid function recovered after treatment. *Blautia*, *Veillonella*, *Streptococcus*, *Ruminococcus* and *Lactobacillus* were enriched in GD, while *Phascolarctobacterium* and *Synergistetes* were consumed in large quantities. The current research reveals that immune responses against gut microflora could be of great relevance to the regulation of TRAb observed in GD patients, with the decreased *Synergistetes* and *Phascolarctobacterium* probably have a protective role in the pathogenesis of GD, while the increased *Ruminococcus* and *Lactobacillus* possibly play a role in the immune mechanism of Graves' disease. Our findings provide a few references for elucidating the mechanism of gut microbiota disorder in Graves' disease. Nevertheless, there are still many problems needed to be solved and more further studies needed to be conducted.

Table 3 Spearman correlation analysis of gut microbiota and immune indexes between HG and CG (*P*, *R*)

FLORA	TRAb	TSH	TGAB	TPOAB
Firmicutes	0.365, -0.208	0.313, 0.194	0.326, -0.201	0.733, -0.072
Actinobacteria	0.275, 0.25	0.585, -0.106	0.324, 0.233	0.126, -0.364
Bacteroidetes	0.908, 0.027	0.789, -0.052	0.479, -0.168	0.954, -0.014
Proteobacteria	0.581, -0.128	0.543, 0.118	0.289, -0.249	0.532, -0.153
Synergistetes	0.000* , -0.702	0.001* , 0.594	0.003* , -0.624	0.001* , -0.711
Lactobacillus	0.024* , 0.489	0.001* , -0.565	0.061, 0.427	0.006* , 0.607
Veillonella	0.572, 0.131	0.05, -0.367	0.424, 0.189	0.208, 0.302
Streptococcus	0.269, 0.253	0.284, -0.206	0.346, 0.222	0.347, 0.228
Phascolarctobacterium	0.011* , -0.544	0.042* , 0.38	0.077, -0.405	0.160, -0.336
Coprococcus	0.103, -0.366	0.026, 0.412	0.251, -0.269	0.030* , -0.499

P < 0.05 indicates that the results are statistically significant. The obtained data is represented by *P* value and *R* value (*P*, *R*)

Table 4 Spearman correlation analysis of gut microbiota and immune indexes between HG and TG (*P*, *R*)

FLORA	TRAb	ΔTRAB	TGAB	TPOAB
Blautia	0.246, 0.298	0.926, 0.032	0.968 , -0.01	0.616, -0.131
Corynebacterium	0.21, 0.32	0.152, 0.462	0.698, 0.098	0.630, -0.126
Ruminococcus	*0.021 , 0.553	*0.035 , 0.638	0.964, 0.011	0.694, 0.103
TM7	0.885, -0.038	0.858, -0.061	0.106, -0.394	0.699, 0.101
Sutterella	0.642, -0.122	0.164, -0.451	0.887, 0.036	0.907, -0.031
Haemophilus	0.713, 0.096	0.905, -0.041	0.06, 0.452	0.598, 0.138
Oscillospira	0.809, -0.063	0.894, -0.046	*0.007 , -0.609	0.264, -0.287
Lachnospira	0.117, -0.395	0.441, -0.260	0.192, 0.322	0.822, 0.059
Streptococcus	0.138, 0.375	0.228, 0.396	0.263, 0.279	0.804, -0.065
Actinobacteria	0.147, 0.367	0.264, 0.369	0.665, 0.110	0.340, -0.246
Veillonella	0.861, 0.046	0.527, 0.214	*0.02 , 0.544	0.593, -0.14
Lactobacillus	0.517, 0.169	0.526, 0.215	0.704, -0.096	0.687, 0.105
Bacteroidetes	0.865, -0.045	0.164, -0.451	0.232, -0.296	0.797, -0.067
Proteobacteria	0.985, -0.005	0.228, -0.396	0.416, -0.205	0.874, -0.042
Phascolarctobacterium	0.065, -0.457	*0.046 , 0.610	0.064, -0.445	0.224, -0.311

The correlation between the difference value of clinical variables and that of flora abundance in patients with GD before and after treatment was analyzed, and the correlation was not observed in TGAB and TPOAB, so it was not listed in the table, while the difference of TRAB was expressed as ΔTRAB

Author contributions CJ, SJQ and GP contributed to the conception and design of this study. Data preparation, sample collection, data collection and analysis were conducted by CJ, WW, GZH, HSS, LHY, ZP and LB. The first draft of the paper was written by CJ, and all authors participated in revising the previous version of the manuscript. All the authors have read and approved the final paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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