Alterations of Gut Microbiota in Patients With Graves’ Disease

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Graves’ disease (GD) is a systemic autoimmune disease characterized by hyperthyroidism. Evidence suggests that alterations to the gut microbiota may be involved in the development of autoimmune disorders. The aim of this study was to characterize the composition of gut microbiota in GD patients. Fecal samples were collected from 55 GD patients and 48 healthy controls. Using 16S rRNA gene amplification and sequencing, the overall bacterial richness and diversity were found to be similar between GD patients and healthy controls. However, principal coordinate analysis and partial least squares-discriminant analysis showed that the overall gut microbiota composition was significantly different (ANOSIM; p < 0.001). The linear discriminant analysis effect size revealed that Firmicutes phylum decreased in GD patients, with a corresponding increase in Bacteroidetes phylum compared to healthy controls. In addition, the families Prevotellaceae, and Veillonellaceae and the genus Prevotella_9 were closely associated with GD patients, while the families Lachnospiraceae and Ruminococccaceae and the genera Faecalibacterium, Lachnospira, and Lachnospiraceae NK4A136 were associated with healthy controls. Metagenomic profiles analysis yielded 22 statistically significant bacterial taxa: 18 taxa were increased and 4 taxa were decreased. Key bacterial taxa with different abundances between the two groups were strongly correlated with GD-associated clinical parameters using Spearman’s correlation analysis. Importantly, the discriminant model based on predominant microbiota could effectively distinguish GD patients from healthy controls (AUC = 0.825). Thus, the gut microbiota composition between GD patients and healthy controls is significantly different, indicating that gut microbiota may play a role in the pathogenesis of GD. Further studies are needed to fully elucidate the role of gut microbiota in the development of GD.

Keywords: Graves’ disease, gut microbiota, clinical parameters, 16S rRNA, next-generation sequencing
INTRODUCTION

Graves’ disease (GD) is an autoimmune disorder in which stimulatory autoantibodies activate thyroid stimulating hormone receptors (TSHR), leading to excessive amounts of thyroid hormones. It is the most common cause of hyperthyroidism, with typical symptoms including weight loss, fatigue, heat intolerance, tremor, and palpitations. GD can occur at all ages, but is more common in women than in men (Ehlers et al., 2019). The prevalence of hyperthyroidism in the United States is approximately 1.2% (0.5% overt and 0.7% subclinical) (Doubleday and Sippel, 2020). The prevalence rate of overt hyperthyroidism estimated by a population-based study in Taiwan is around 0.27-0.37%, with an annual incidence of 0.97 to 1.06 cases per 1,000 individuals (Kornelius et al., 2018). The pathogenesis of GD remains unclear, but it is hypothesized that infections, iodine intake, smoking, and mental stress play a role, and both genetic and environmental factors appear to be involved (Shukla et al., 2018).

The human gut microbiota harbors up to 100 trillion microbes. These microbes play a crucial role in the maintenance of human health by affecting nutrient digestion, immune response, metabolic homeostasis, and protection against pathogen colonization (Lin and Zhang, 2017; Pickard et al., 2017). An imbalance in the gut microbiota or dysbiosis can increase epithelial barrier dysfunction and lead to intestinal and systemic disorders. Recent studies have established a link between the alterations in the composition of gut microbiota in many disease states, including inflammatory bowel disease, Crohn’s disease, obesity, and type 2 diabetes (Ortega et al., 2020; Szilagyi, 2020). In addition, the components and metabolites derived from the gut microbiota are key regulators of host immune responses. Alterations in the gut microbiota may result in an imbalance of T-cell subpopulations, triggering the release of pro-inflammatory and anti-inflammatory cytokines (Lee and Kim, 2017). Moreover, this can trigger the onset of several types of autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus (Alkanani et al., 2015; Correa et al., 2017; Picchiante-Diamant et al., 2018).

The thyroid gland is the primary endocrine gland in the human body. However, the relationship between the gut microbiota and GD remains unclear. The aim of this study was to characterize the changes in the gut microbiota of patients with GD using 16S rRNA gene sequencing, compare it with the gut microbiota of their healthy counterparts, and determine its possible correlation with the relevant clinical factors.

MATERIALS AND METHODS

Ethics Statement

This study’s protocol was approved by the Institutional Review Board and Ethics Committee of Chang Gung Memorial Hospital (IRB 201701806B0C502). Written informed consent was obtained from all participants before the start of the study. All experimental procedures were performed in accordance with the approved guidelines.

Study Population and Sample Collection

A total of 55 GD patients were recruited from the Division of Endocrinology and Metabolism at Chang Gung Memorial Hospital between October 2017 and March 2020. These patients were previously diagnosed with GD and had an average follow-up of 45.33 months. All patients were initially treated with anti-thyroid drugs, including propylthiouracil (PTU), methimazole (MMI), or carbimazole (CBZ). GD was diagnosed on the basis of clinical presentations and laboratory data, including symptoms and signs of thyrotoxicosis, diffuse goiter, the presence of ophthalmopathy, elevated free T4 and suppressed TSH levels, and the presence of autoantibody against TSH receptor. In addition, 48 age-, sex-, and BMI-matched healthy subjects from a health screening center constituted the control group. The control group had normal values of free T4, TSH, and thyroid peroxidase (TPO) antibody, and no history of thyroid disease. Free T4 and TSH levels were measured using a one-step chemiluminescent immunoassay (CLIA) on the ADVIA Centaur XP system (Siemens Healthcare, Munich, Germany). TPO antibody and TSH receptor antibody were measured by chemiluminescent microparticle immunoassay (CMIA) (Architect, Abbott, Wiesbaden, Germany). Reference intervals were defined as follows: free T4 0.70-1.48 ng/dL; TSH 0.35-5.50 µIU/mL; anti-TPO <5.61 IU/mL; anti-TSHR <1.75. A questionnaire was used to assess dietary habits and health condition of all subjects. In each group, subjects who met any of the following criteria were excluded from this study: pregnancy; gastrointestinal disorders (chronic diarrhea or inflammatory bowel disease); gout; stroke; cancer; autoimmune diseases (type 1 diabetes, systemic lupus erythematosus, or rheumatoid arthritis); history of gastrointestinal surgery; use of antibiotics, probiotics, prebiotics, symbiotics (<2 months), hormonal medication or Chinese herbal medicine (<3 months); and pure vegetarian. The fecal samples were collected in a clean container, aliquoted, and all aliquots were immediately frozen and stored at -80°C.

16S rRNA Gene Sequencing

DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) following the manufacturer’s protocol. Amplification of the 16S V3-V4 region was performed using the universal primers reported previously (Klinworth et al., 2013). Samples were amplified using 2× KAPA HiFi HotStart Ready Mix (KAPA Biosystems, MA, USA), and indexed using the Nextera XT 96 Index Kit (Illumina, CA, USA). Lastly, the library was sequenced on an Illumina MiSeq platform and 300 bp paired-end reads were generated.

Bioinformatics and Statistical Analysis

The overlapping and merging of paired-end reads were conducted in FLASH (v1.2.11) (Magoc and Salzberg, 2011). Filtering of the raw tags was performed under specific filtering conditions according to the QIIME (v1.9.1) (Caporaso et al., 2010). Chimeras were removed by aligning sequences to the “gold” reference database using UCHIME (Edgar et al., 2011). The resulting filtered sequences were clustered into operational
taxonomic units (OTUs) using UPARSE (v7.0.1090) with a 97% similarity threshold. The representative sequences were then classified taxonomically using the ribosomal database project (rDP) classifier (v2.2) with default 0.8 as the confidence threshold. The OTUs were aligned using PyNAST (v1.2) and assigned to taxonomic groups against the Greengenes database (v13.8) or SILVA database (v132) (Quast et al., 2013). The alpha diversity metrics were estimated in QIIME (v1.9.1), including the coverage percentage (Good’s), Venn diagram, richness estimators (ACE and Chao1), and diversity indices (Shannon and Simpson). Rank-abundance and rarefaction curves were calculated at a level of 97% similarity of the OTUs. Beta diversity measures, such as principal coordinate analysis (PCoA) and partial least squares-discriminant analysis (PLS-DA), were calculated using QIIME (v1.9.1). The bacterial taxonomic distributions of the sample communities were visualized using R software. Venn diagrams were plotted using the VennDiagram package in R. Significant differences in the microbiota structure between the two groups were evaluated by nonparametric analysis of similarity (ANOSIM) using the vegan package in R. The specific characterization of the gut microbiota that differentiate among the groups was analyzed using a linear discriminant analysis (LDA) effect size (LEfSe) algorithm (Segata et al., 2011). The parameters set with default p-value, α = 0.05, and an LDA score of 4.0 with LEfS. The relative abundances of OTUs between the GD group and the healthy controls were compared using Statistical Analysis of Metagenomic Profiles (STAMP) software (v2.1.3), Welch’s t-test (p < 0.05), and Benjamini–Hochberg False Discovery Rate (FDR) method (q < 0.05) as a multiple test correction (Parks et al., 2014). The correlation analysis between clinical parameters and different microbiota was performed using Spearman rank correlation, where a p-value < 0.05 was considered statistically significant. Random forest algorithm was used to evaluate the prediction accuracy of the gut microbiota of GD patients and healthy controls. The receiving operational curve (ROC) was analyzed and the area under curve (AUC) using ten-fold cross-validation was calculated. Anthropometric and clinical characteristics were analyzed using SPSS version 18.0 (SPSS Inc., Chicago, USA).

RESULTS

Study Population

Fifty-five subjects were enrolled into the GD group and 48 volunteers were enrolled into the healthy control group. The demographic details of the subjects are summarized in Table 1. As expected, the GD subjects had a higher FT4 (p = 1.98 × 10^{-5}) and TPOAb levels (p = 1.26 × 10^{-10}) compared to the control subjects. The GD subjects also had lower TSH (p = 3.73 × 10^{-11}). There were no significant differences in age, sex, or body mass index (BMI) (p > 0.05).

### Table 1 | Clinical and demographic characteristics of GD patients and healthy controls.

| Parameters | GD (N=55) | Control (N=48) | p-value |
|------------|-----------|---------------|---------|
| Sex (M/F)  | 20/35     | 18/30         | 0.905   |
| Age (years) | 45.09 ± 12.08 | 42.60 ± 9.78 | 0.258   |
| BMI (kg/m²) | 23.84 ± 4.20 | 23.27 ± 3.41 | 0.459   |
| FT4 (ng/mL) | 2.25 ± 1.58 | 1.22 ± 0.14 | 1.26x10^{-5} |
| TSH (mIU/mL) | 0.40 ± 0.83 | 0.48 ± 0.60 | 3.73x10^{-11} |
| TPOAb (IU/mL) (P/N) | 55/0 | 55/0 | 0.909303 |

GD, Graves’ disease; M/F, male/female; BMI, body mass index; FT4, free thyroxine; TSH, thyrotropin; TPOAb, thyroperoxidase antibody; P/N, positive/negative ratio. Values are expressed as mean ± standard deviation.

#### Table 2 | Phylotype coverage and diversity estimation of the 16S rRNA gene libraries at 97% similarity from the MiSeq analysis.

| Group | No. of reads | No. of OTUsa | Good’s (%)b | Richness estimators | Diversity index |
|-------|--------------|--------------|-------------|---------------------|----------------|
|       |              |              |             | ACE mean 95% C.I.   | Shannon mean 95% C.I. |
|       |              |              |             | Chao1 mean 95% C.I. | Simpson mean 95% C.I. |
| Control | 3,570,066   | 671          | 0.9985      | 196.89 182.4568-214.8318 | 4.5097 0.889637 |
| GD     | 2,213,210   | 684          | 0.9985      | 196.98 188.8540-217.0350 | 4.8725 0.909303 |

a The operational taxonomic units (OTUs) were defined at 97% similarity level.
b The coverage percentage (Good’s), richness estimators (ACE and Chao1), and diversity indices (Shannon and Simpson) were calculated using Good’s method and the QIIME pipeline (v1.9.1), respectively. C.I., confidence interval.
FIGURE 1 | The gut microbiota of GD patients differs from that of healthy controls. Rank-abundance curves were used to explain species richness and evenness (A). Rarefaction curves were used to evaluate the species richness. The bacterial communities of the healthy controls (HC) and GD patients exhibited similar patterns (B). The relative abundances of the top 10 microbial phyla and genera are represented. Only phyla and genera present at relative abundances >1% are shown. Taxa with lower abundances are grouped as “other” (C, D). Principal coordinates analysis (PCOA) based on the distance matrix of Bray-Curtis dissimilarity at the OTU level showed that the gut microbiota of GD patients was separated clearly from those of healthy controls (E). The PLS-DA (Partial Least Squares Discriminant Analysis) showed a significant separation between GD and healthy controls (F).
the two groups, the unique and overlapping OTU data were presented in a Venn diagram. These diagrams showed that 589/766 OTUs were shared between the two groups. Thus, 95 and 82 unique OTUs were identified in the GD group and healthy controls, respectively (Supplementary Figure S1).

**Differences in Gut Microbiota Composition Between GD and Healthy Controls**

To further analyze the composition of the gut microbiota in the different experimental groups, taxonomy from phylum to genus level was assigned to each OTU (Supplementary Figure S2). As a result, the relative abundance of the phyla Bacteroidetes and Actinobacteria was found to increase, while Firmicutes decreased in the GD group compared to the healthy controls (Figure 1C). In addition, at the genus level, the abundances of Bacteroides and Prevotella_9 were found to be significantly higher, while Faecalibacterium and Lachnospiraceae_NK4A136_group were slightly lower in the GD group compared to the healthy controls (Figure 1D). To measure similarities between the microbial communities, beta diversity analysis was performed using principal coordinate analysis (PCoA) and partial least squares-discriminant analysis (PLS-DA). Despite significant inter-individual variations, the gut microbiota from GD group were clearly different from those of the healthy controls (Figure 1E). A clear separation between groups was also observed in PLS-DA (Figure 1F). The results of analysis of similarities (ANOSIM) indicated that the structure of the gut microbiota significantly differed between the two groups (ANOSIM, r = 0.158, p < 0.001). This comparison revealed that the bacterial diversity of the gut microbiota in the GD group was similar to that observed in the healthy controls. However, the overall community structure was distinctive between the two sample groups.

To identify the specific bacterial taxa associated with GD patients, the gut microbiota in the healthy controls and GD patients was compared using the LEfSe method. A cladogram representing the structure of the gut microbiota and the predominant bacteria in the healthy controls and GD patients is shown in Figure 2A, in which the greatest differences in the taxa between the two communities are displayed. LEfSe analysis revealed 16 discriminative features, including 2 phyla, 3 classes, 3 orders, 4 families, and 4 genera (LDA score >4; Figure 2B). Bacteria belonging to the Bacteroidetes phylum were enriched in the GD patients, while those in Firmicutes were more abundant in the healthy controls. At the family level, the proportion of Prevotellaceae and Veillonellaceae was increased in GD patients, while that of Lachnospiraceae and Ruminococcaceae was decreased compared to that in healthy controls. Moreover, the level of Prevotella_9 was significantly higher in GD patients; however, the levels of Faecalibacterium, Lachnospira, and Lachnospiraceae_NK4A136_group were markedly increased in the healthy controls. Thus, these microbial features may be used as potential biomarkers for distinguishing GD patients from healthy controls.

**Correlation Between Clinical Parameters and the Gut Microbiota**

To explore the association between the clinical parameters and gut microbiota, a Spearman’s correlation analysis was conducted among five clinical parameters (TSH, TPOAb, FT4, age, and BMI) with 29 bacterial taxa, which were differentially abundant between the GD group and healthy controls in the LEfSe and STAMP analyses (Figure 2). The abundance levels of 16 GD-enriched taxa were positively correlated with TPOAb but negatively correlated with TSH, while six taxa enriched in the healthy controls showed contrasting correlations (p < 0.05) (Figure 3A). Among these, 12 out of 16 GD-enriched taxa were also positively correlated with FT4, while three of the six control-enriched taxa were also negatively correlated with FT4 (p < 0.05). However, there were no significant correlations between the gut microbiota and other environmental factors, including age and BMI (p > 0.05). A heatmap was generated based on the 15 most abundant taxa from the 29 different taxa. Hierarchical clustering showed a clear separation between the GD group and the healthy controls (Figure 3B). Further, to evaluate the predictive power of these taxa in predicting the GD status, the random forest analysis was used and an AUC value of 0.825 was achieved (Figure 3C), indicating that these microbial features may be used as potential biomarkers for distinguishing GD patients from healthy controls.

**DISCUSSION**

In this study, the gut microbiota of GD patients was compared with those of age- and sex-matched healthy subjects using 16S rRNA sequencing technologies. Patients with GD were found to have an altered gut microbiota composition compared to the healthy controls. In fact, differentially abundant bacterial taxa responsible for the differences between the two groups were identified. In addition, Spearman correlation analysis confirmed that several taxa were significantly correlated with clinical parameters. Among these, the top 15 most abundant taxa discriminated GD patients with high accuracy (AUC = 0.825).

Fecal samples from GD patients were found to show a slight increase in microbial diversity and richness compared to those of the healthy controls, although the differences were not statistically significant (Table 2). Previous studies have
observed bacterial diversity in hyperthyroid and hypothyroid patients, which may be associated with bacterial overgrowth in the intestinal tract (Lauritano et al., 2007; Zhou et al., 2014). Recently, increased microbial diversity has also been reported in patients with Hashimoto’s thyroiditis and thyroid carcinoma (Zhao et al., 2018; Feng et al., 2019).

PCoA and PLS-DA analyses demonstrated that the microbial compositions of GD patients and healthy controls were divided into two separate groups; the GD group exhibited a relatively high homology, indicating that GD patients may share common microbiota features (Figures 1E, F). In our study, GD patients had significantly higher levels of *Bacteroidetes* and *Actinobacteria* and lower levels of *Firmicutes* compared to the healthy controls (Figure 2 and Table 3). These findings are consistent with those of previous studies in patients with GD and Graves’ orbitopathy (Shi et al., 2019; Jiang et al., 2021). However, in other studies of patients with Hashimoto’s thyroiditis or thyroid cancer, *Firmicutes* was found to be more abundant and *Bacteroidetes* less abundant (Zhao et al., 2018; Feng et al., 2019). The ratio of *Firmicutes* to *Bacteroidetes* has been considered as an index of the health of the gut microbiota and has been correlated with obesity (Mariat et al., 2009; Tilg and Kaser, 2011). However, a recent meta-analysis suggested that the way in which the gut microbiota modulates obesity is more complex than a simple imbalance in these commensal phyla.

In addition, our study showed that the fecal samples of the GD patients were rich in members of the species *Actinomyces odontolyticus*, the genus *Collinsella*, *Parabacteroides*, and *Prevotella* 9, as well as of the family *Veillonellaceae* (Figures 2B, C). *Actinomyces odontolyticus* is normally present in the oral cavity and gastrointestinal tract of healthy humans. This species has been shown to be involved in the formation of biofilms on teeth (Liljemark et al., 1993), and has been associated with several human diseases, including actinomycosis, periodontitis, and colorectal cancer (Ximenez-Fyvie et al., 1999; Riegert-Johnson et al., 2002; Yachida et al., 2019). Although its role in the pathogenesis of inflammatory bowel disease has not been demonstrated, it may change the enteric environment and immune factors and aggravate...
TABLE 3 | Significant differences in taxa abundances from phylum to species level between GD patients and healthy controls.

| Taxonomic level | Bacterial taxa                    | GD mean  | Control mean | p-value   | q-value   |
|-----------------|-----------------------------------|----------|--------------|-----------|-----------|
| Phylum          | Actinobacteria                    | 2.88     | 1.11         | <0.01     | <0.01     |
| Class           | Bacteroidetes                     | 42.1     | 26.9         | <0.01     | <0.01     |
|                 | Firmicutes                        | 50.2     | 66.3         | <0.01     | <0.01     |
| Order           | Bacteroidetes                     | 42.1     | 26.9         | <0.01     | <0.01     |
|                 | Clostridia                        | 43.8     | 64.1         | <0.01     | <0.01     |
|                 | Coriobacteria                     | 0.653    | 0.18         | <0.01     | <0.01     |
|                 | Erysipelotrichia                  | 1.56     | 0.31         | <0.01     | <0.01     |
| Family          | Actinomycetaceae                  | 7.17x10^-3  | 7.92x10^-4     | <0.01     | <0.01     |
|                 | Coriobacteriaceae                 | 0.535    | 0.142        | <0.01     | <0.01     |
|                 | Erysipelotrichiaceae              | 1.56     | 0.31         | <0.01     | <0.01     |
|                 | Lachnospiraceae                   | 16.9     | 26.6         | <0.01     | <0.01     |
|                 | Prevotellaceae                    | 10.9     | 1.42         | <0.01     | <0.01     |
|                 | Tannerellaceae                    | 1.1      | 0.085        | <0.01     | <0.01     |
| Genus           | Collinsella                       | 0.523    | 0.142        | <0.01     | <0.01     |
|                 | Parabacteroides                   | 1.1      | 0.085        | <0.01     | <0.01     |
|                 | Prevotella_9                      | 7.67     | 0.166        | <0.01     | 0.024     |
| Species         | Actinomyces_odontolyticus         | 6.83x10^-3  | 6.93x10^-4     | <0.01     | 0.017     |

Significant differences between groups are shown as \( p < 0.05 \) and \( q < 0.05 \), according to Welch’s t-test.
correlated with GD. Nevertheless, this study has several limitations. First, this was a single-center, cross-sectional study involving a limited number of patients. Second, whether the altered gut microbiota is a cause or a consequence of GD development will need to be confirmed. Lastly, gut microbiota studies using 16S rRNA sequencing have yet to provide a detailed list of functional genes and metabolic pathways for the study of host–gut microbiome interactions. Therefore, further multicenter studies with larger samples using metagenomics and metabolomics integrative analyses are needed to improve our understanding of the interactions between gut microbes, their metabolites, and host cells.

DATA AVAILABILITY STATEMENT
The datasets presented in this study can be found in online repositories. The names of the repository/repositories and...
accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA695309.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board and Ethics Committee of Chang Gung Memorial Hospital (IRB 201701806B0C502). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

S-CC, S-FL, and J-JL designed the experiments. S-FL and S-TC collected the samples and clinical data. P-YC and F-SL helped supervise the project. Y-MY performed the statistical analyses. S-CC and J-JL wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors contributed to the article and approved the submitted version.

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FUNDING

This work was supported by grants from the Chang Gung Memorial Hospital (CMRPG3F1693).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.663131/full#supplementary-material

Supplementary Table 1 | Comparison of phylotype coverage and diversity estimation of the 16S rRNA gene libraries at 97% similarity from the sequencing analysis. GD, Graves’ disease; HC, healthy controls.

Supplementary Figure 1 | Venn diagram illustrating the overlap of OTUs in the gut microbiota among the two groups

Supplementary Figure 2 | Composition analysis of the gut microbiota at different taxonomic levels among the two groups: (A) phylum, (B) class, (C) order, (D) family, (E) genus. Only the top 10 most abundant OTUs are displayed.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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