Gut microbiota profiling in Han Chinese with type 1 diabetes

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ABSTRACT

Aims: To investigate whether microbial dysbiosis is associated with T1D in Chinese population and to explore relationships between the composition of gut microbiome and clinical data.

Methods: In this study, we recruited 10 healthy and 12 T1D Han Chinese subjects between the ages of 12 to 33. Fecal samples were collected for DNA extraction and 16S rRNA sequencing, followed by analyses of the gut microbiota composition.

Results: Bacterial communities differed between healthy and T1D subjects. At the phylum level, Bacteroidetes and Firmicutes are the dominant phyla in T1D patients and healthy controls, respectively. The linear discriminant analysis (LDA) effect size (LEfSe) algorithm detected 28 bacterial taxonomic clades showing statistically differences (13 increased and 15 decreased) in T1D patients. Association analyses of clinical data and microbial community abundance demonstrated that abundances of Faecalibacterium were negatively correlated with HbA1c levels ($Z = -2.614, P = 0.017$). The numbers of detected anti-islet cell autoantibodies were positively correlated with Bacteroides ($Z = 2.531, P = 0.011$) and Bilophila ($Z = 2.477, P = 0.013$) abundances, while negatively correlated with abundances of Streptococcus ($Z = -2.041, P = 0.041$) and Ruminococcaceae ($Z = -2.23, P = 0.026$).

Conclusions: These results suggest that Han Chinese T1D patients possess distinctly different gut microbiota, compared to healthy subjects, characterized by increased Bacteroidetes/Firmicutes ratio, negative correlation of Faecalibacterium abundance with HbA1c, and positive correlation of Bacteroides abundance with the presence of autoantibodies.

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1. Introduction

The microbial community, resides in various anatomical areas of the human body. A large body of research has demonstrated that the microbiota, as a "hidden organ", has significant impacts on many aspects of the host physiology, including metabolism, immunity, and neurophysiology [1,2]. The symbiotic microbiota-host interactions ensure...
appropriate development and function of the metabolic system [3]. On the other hand, microbiota dysbiosis, which is defined by the imbalance of microbial composition and structure is associated with and contributes to the pathogenesis of many metabolic diseases, such as obesity, type 1 and type 2 diabetes (T1D and T2D), liver diseases, gastrointestinal disorders, and cardiovascular diseases [4–8]. With recent advances in high-throughput sequencing, integrative multi-omics data analyses, and gnotobiotic animal models, we have gained substantial knowledge about the establishment and environmental modulation of microbiota, microbiota-host interactions in physiology and pathology, and microbiota-targeted diagnostic and therapeutic applications.

T1D, characterized by immune-mediated self-destructing of pancreatic β cells, is caused by both genetic and environmental factors. Risk alleles in human leucocyte antigen (HLA) genes are considered strongest genetic factor for T1D; however, only around 5% of children carrying these alleles develop T1D, implicating gene-environment interactions in the pathogenesis of T1D [9]. Gut microbiota has long been proposed to contribute to the etiology of T1D. In non-obese diabetic (NOD) mice, the incidence of T1D is higher when mice are raised in specific-pathogen-free (SPF) facilities than in conventional conditions [10], while antibiotic-mediated gut microbial perturbation accelerates T1D development [11–13]. Moreover, fecal microbiota transplantation from NOD to non-obese resistant mice produces insulitis, which can also be accelerated by antibiotics [13]. These data suggest that microbial exposure, especially during early life, is critical for the protection from autoimmune diabetes in mice.

In patients with T1D, gut microbiobial dysbiosis has been shown by a few studies to be associated with the progress of T1D [14–20], and mostly carried out in Caucasians. Although the incidence of T1D in China is low, the number of people suffering from the disease is still increasing rapidly over the past decade [22], which suggests non-genetic factors contribute to the epidemics. It is still unknown if microbial dysbiosis is associated T1D in Chinese population. Here, we performed 16S rDNA sequencing and analyses of gut microbiota in young healthy subjects and T1D patients from the Han ethnic group.

2. Subjects

2.1. Study population

The study protocol was approved by the Institutional Review Board of the Second Affiliated Hospital of Soochow University. We recruited 12 subjects who were diagnosed T1D according to the criteria of the American Diabetes Associations and 10 healthy subjects. The study participants were excluded if they met one of the following criteria: acute or chronic inflammatory diseases or infectious diseases, chronic gastrointestinal disease, receiving antibiotic treatment within 3 months before the enrollment or receiving other treatments including probiotics and prebiotics, use of corticosteroids, breastfeeding or pregnancy. Details such as personal and treatment history were also recorded.

2.2. Sample collection

A total of 22 fecal samples (12 T1D patients and 10 healthy controls) were collected in a sterile container, brought to the laboratory, and kept at −80 °C until processing. Samples were analyzed in the central laboratory of the Second Affiliated Hospital of Soochow University using routine procedures for levels of hemoglobin A1c (HbA1c), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL) cholesterol.

3. Materials and methods

3.1. Autoantibody detection

Glutamic acid decarboxylase autoantibodies (GADA) were measured by enzyme-linked immunosorbent assay kit (Biomerica Inc. USA). The protein tyrosine phosphatase-like protein (IA2) and Zinc transporter 8 (ZnT8) were measured by radiobinding assays. Human 35S-labelled recombinant antigens were produced in an in vitro-coupled transcription and translation system with SP6 (for IA2) or T7 (for ZnT8) RNA polymerase and nuclease-treated rabbit reticuloocyte lysate (Promega, Biotech Co. Beijing). Sera (5 μL) were incubated with 35S-antigens (≥ 20 000 of TCA-precipitable radioactivity). After an overnight incubation at 4 °C, antibody-bound 35S-antigens were separated from unbound antigen by precipitation with Protein A Sepharose (GE Amer sham Biosciences). The immunoprecipitated radioactivity was counted on a Wallac Microbeta Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences).

3.2. DNA extraction, PCR and 16S sequencing

Genomic DNA from stool sample was extracted using the E.Z. N.A. Mag-Bind Soil DNA Kit (Omega Bio-tek), following manufacturer’s instructions. DNA concentration and purity were evaluated using a Qubit 2.0 Fluorometer (ThermoFisher Scientific). The V3-V4 region the bacterial 16S rRNA gene was amplified using 341F/805R primers fused with adaptors and barcodes. V3-V4 amplicons were purified using an Agen courtAMPure XP system (Beckman Coulter) and quantified with a Qubit 2.0 Fluorometer. Samples were pooled in equal quantities to form the sequencing library. Paired-end IlluminaMiSeq sequencing was then performed at Sangon Biotech.

3.3. Bioinformatics and statistics

The sequence analysis was performed mainly with QIIME [23]. Low-quality sequences were eliminated from analysis based on the following criteria: (1) raw reads shorter than 50 bp; (2) sequences with low complexity; (3) sequences with mismatch ratio higher than 0.1 in the overlap region. Sequence assembly and data quality control were implemented in FLASH and Prinseq, respectively [24,25]. Mothur was used to remove erroneous and chimeric sequences [26]. Closed-
reference Operational Taxonomic Units (OTU) picking was performed using Uclust and taxonomy was assigned using the RDP classifier [27,28]. The relative abundance of each OTU was determined as a proportion of the sum of sequences for each sample. Taxonomic relative abundance profiles at the phylum, class, order, family and genus levels were generated based on OTU annotation. Alpha-diversity including richness and evenness was calculated using Mothur. Unweighted UniFrac distances and Principal coordinate analyses were generated using QIIME.

To determine different taxa between healthy and T1D, the linear discriminant analysis (LDA) effect size (LEfSe) algorithm was performed on the Galaxy website [29]. Linear (for age, BMI, HbA1c, TC, TG, LDL, VLDL, and HDL) and logistic (for gender, GADA, IA2, and Znt8) regression analyses were performed to identify the biological covariates which were associated with different bacterial taxa. Poisson regression was used to analyze the association between bacterial taxa and total numbers (0, 1, 2, or 3) of detected autoantibodies.

# Results

## 4.1. Microbiota diversity in T1D patients

The study population was comprised of 12 T1D patients and 10 healthy subjects. All subjects were antibiotic-free for at least 3 months at the time of study. All T1D patients were receiving insulin treatment and HbA1c levels were less than 9.0% (75 mmol/mol). Control subjects had an average HbA1c of 5.2% (33 mmol/mol) which was significantly lower than that of T1D patients (p < 0.001, Table 1). Seven out of the 12 T1D patients were positive for at least one of the three islet autoantibodies, while none of the control subjects were positive (p < 0.001). Gender proportion, age, BMI and cholesterol levels did not differ between two groups.

Sequencing of the V3-V4 region of the 16S rRNA gene was performed, with a mean sequencing depth of 34,429 reads per sample. The alpha diversity of the microbiota, estimated by the total number of OTU, Chao index, Shannon index, and Simpson index, was not significantly different between controls and T1D patients (Fig. S1A-D).

## 4.2. Increased Bacteroidetes/Firmicutes ratio in T1D gut microbiota

Principal coordinates analysis of the beta diversity showed a separate clustering of healthy controls and T1D patients (Fig. 1A). Taxonomic analyses demonstrated that microbial composition was significantly different between two groups at the phylum level (Fig. 1B). The top 5 phyla in T1D and controls were Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria and Verrucomicrobia which constituted 99.3–99.9% microbiota in two groups. In T1D patients, the dominant phylum was Bacteroidetes; while in healthy controls, Firmicutes was the dominant phylum (Fig. 1B).

To determine different taxa between two groups, the linear discriminate analysis (LDA) effect size (LEfSe) algorithm on the Galaxy browser was used. LEfSe detected 28 bacterial taxonomic clades showing statistically significant differences (13 increased and 15 decreased) in T1D patients, compared to healthy controls (Fig. 2A). At the family level, the most differentially abundant bacteria in healthy subjects included Ruminococcaceae, Veillonellaceae, Phascolarctobacterium, and Paenibacillaceae, which all belong to Firmicutes, while Porphyromonadaceae, a family of Bacteroidetes, was overrepresented in T1D patients (Fig. 2B). In addition, the phylum Fusobacteria was differentially enriched in healthy subjects (Fig. 2B).

## 4.3. Correlations between the gut microbiome and clinical data

We performed regression analyses to assess the association between the gut microbiome and clinical data. Linear regression was used for BMI, and levels of HbA1c, TC, TG, LDL, VLDL, and HDL, and logistic regression for gender, GADA, IA2, and Znt8. Poisson regression was used to analyze the association between bacterial taxa and total numbers (0, 1, 2, or 3) of detected autoantibodies.

### Table 1 - Clinical data of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects (n = 10)</th>
<th>T1D patients (n = 12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>5/5</td>
<td>5/7</td>
<td>0.29</td>
</tr>
<tr>
<td>Age (year)</td>
<td>25.10 ± 0.41</td>
<td>22.58 ± 2.24</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.78 ± 0.61</td>
<td>20.89 ± 1.31</td>
<td>0.94</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.24 ± 0.10</td>
<td>7.18 ± 0.35</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>C-peptide (ng/mL)</td>
<td>ND</td>
<td>0.44 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>3.60 ± 0.02</td>
<td>4.55 ± 0.34</td>
<td>0.54</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.08 ± 0.12</td>
<td>0.77 ± 0.08</td>
<td>0.046</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.33 ± 0.15</td>
<td>1.60 ± 0.13</td>
<td>0.18</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.01 ± 0.21</td>
<td>2.52 ± 0.23</td>
<td>0.12</td>
</tr>
<tr>
<td>VLDL (mmol/L)</td>
<td>0.42 ± 0.06</td>
<td>0.42 ± 0.06</td>
<td>0.91</td>
</tr>
<tr>
<td>Autoantibodies</td>
<td>0/10</td>
<td>7/12</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>-GADA</td>
<td>0/10</td>
<td>6/12</td>
<td>0.002</td>
</tr>
<tr>
<td>-IA2</td>
<td>0/10</td>
<td>5/12</td>
<td>0.008</td>
</tr>
<tr>
<td>-Znt8</td>
<td>0/10</td>
<td>5/12</td>
<td>0.008</td>
</tr>
</tbody>
</table>

ND, not determined; BMI, body mass index; HbA1c, hemoglobin A1c; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein. Data shown as mean ± SEM.
and HDL, while logistic regression was applied to gender and autoantibodies. The taxa that were detected in over 50% of the subjects (n > 10) were included. We found that the abundances of bacterial taxa including Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria were associated with gender, BMI, and serum LDL, TC, TG, VLDL levels, but none were significantly different between healthy controls and T1D patients (Table S1).

We found that in T1D patients, the abundances of only one family, Ruminococcaceae, and its genus, Faecalibacterium, were negatively correlated with HbA1c levels (Fig. 3A and B).

### 4.4. Autoantibodies and bacterial abundances

In the present study, we determined the associations between bacterial abundances and the presence of three anti-islet cell autoantibodies including GADA, IA2, and ZnT8. As expected, GADA+, IA2+, or ZnT8+ T1D patients showed higher HbA1c levels than healthy controls (Fig. 3A). Poisson regression analyses were performed to identify bacterial taxa associated with the presence of autoantibodies. We found the abundances of the genus Faecalibacterium were significantly higher in GADA+ T1D patients than GADA- subjects (Fig. 3D).

We then calculated the total numbers of autoantibodies in both healthy and T1D subjects, and autoantibody counts were positively correlated with HbA1c levels (Fig. 4A). Poisson regression analyses were performed to identify bacterial taxa associated with the presence of autoantibodies. We found the abundances of the genus Bacteroides were correlated with autoantibody counts (Fig. 4B). The abundances of several taxa in the phylum Firmicutes, including the genus Streptococcus and the family Ruminococcaceae were negatively correlated with autoantibody counts (Fig. 4C and D). In addition, the abundances of the genus Bilophila, which belongs to the phylum Proteobacteria, were positively associated with the numbers of autoantibodies (Fig. 4E).

### 5. Discussion

Commensal bacteria residing in the gut play an important role in the development of the host immune system. In a germ-free condition, mice exhibit impaired immune development and affect the susceptibility to autoimmune diseases including T1D [30]. Administration of broad-spectrum or selective antibiotics to eliminate most bacteria or to perturb microbial composition accelerates T1D development in NOD mice, which is associated with defects in subsets of T cells in the intestinal lamina propria, such as regulatory T (Treg) cells and T helper 17 (Th17) cells [11,12]. On the other hand, exposing laboratory mice to "dirty" wild mice strengthens their immune system and better models the complex immune system in humans [31]. Bacterial antigens and viral infections have been shown to prevent T1D in NOD mice [32]. However, the incidence of diabetes in female NOD mice remained unchanged under germ-free conditions, compared to those housed under SPF conditions [32,33]. These data suggest that gut microbiota not only modulates immune function but also provides insults to induce T1D. Many bioactive microbial products, such as short chain fatty acids (SCFAs), play key functions in microbiota-host interactions. SCFAs are not just an energy source for enterocytes, but also strengthen epithelial barrier function, have profound anti-inflammation activity, and promote peripheral Treg cell generation [34–36]. Abundances of SCFAs-producing bacteria have been shown to be altered in T1D-associated autoimmunity [21].

Our study further proved that gut microbiota in Chinese adult T1D patients is characterized by increased Bacteroidetes/Firmicutes ratio. It agreed with the report carried out in CaucasiansT1D and high-risk cohorts [21]. However, another research in Han Chinese children with T1D showed that no significant difference was found in the phylum level between T1D patients and healthy controls [37]. In our study, we identified the abundances of the Faecalibacterium genus were reduced in T1D and negatively associated with serum levels of Hba1c. The sole known species of this genus, Faecalibacterium prausnitzii, produces butyrate and supports mucosal immune homeostasis [38]. It will be interesting to test if the reduction in the abundance of Faecalibacterium could contribute to the leaky gut in T1D patients, which may lead to a greater exposure of the intestinal immune system to bacterial antigens [39].

In Chinese T1D adult patients, the abundances of the genus Bacteroides, although not significantly changed between healthy and T1D subjects, were positively correlated with the presence of autoantibodies. In the former research of Han Chinese children with type 1 diabetes [37], they found that...
the relative abundance of Blautia, belongs to the order Clostridiales and phylum Firmicutes, was positively correlated with HbA1c, as well as the number of T1DM autoantibodies and the titers of IA-2. Increase in Bacteroides abundance has been demonstrated in several T1D cohorts [21]; however, the functional significance of such change has not been eluci-

Fig. 2 – LEfSe analyses of differentially abundant taxa. (A) A list of taxa that are different between healthy and T1D groups, ranked according to the effect size. (B) Chadogram showing taxonomic representation of differences between healthy and T1D subjects.
dated. It is suggested that the main by-products of the anaerobic respiration in Bacteroides, including acetate and succinate, may compromise epithelial tight junctions and block Treg differentiation [21]. To determine the levels of different SCFAs and immune activation in Chinese T1D patients will be important for future research.

Limitations of this study are mainly reflected in the following 3 points. Firstly, the sample size is relatively small. It should be confirmed in a larger sample and patients of different course (initial and long course) of type 1 diabetes in future. Secondly, the alteration in amount and/or function of T cell subsets needs further investigation to confirm the relationship between imbalance of immune activities and gut microbiota changes. Thirdly, there is no specific and strict restriction on the patients’ and volunteer’s diet before collecting stool samples. However, participants’ dietary habits are relatively close and they had similar situation such as living area, age and income.

In conclusion, this work shows that T1D in Han Chinese is associated with gut microbial imbalance, characterized by increased Bacteroidetes/Firmicutes ratio, negative correlation of Faecalibacterium abundance with HbA1c, and positive correlation of Bacteroides abundance with the presence of autoantibodies. Although not implying causality, this study, combined with future metagenomic, metabolomic, and gnotobiotic analyses will help identify bacterial species and pathways that can be modified to prevent or treat T1D.

**Statement of human and animal rights**

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

**Statement of Informed consent**

Informed consent was obtained from all patients for being included in the study.

**Novelty statement**

This study showed that T1D in Han Chinese is associated with gut microbial imbalance, which might help identify bacterial species and pathways that can be modified to prevent or treat T1D.

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**Fig. 3** – Taxa abundances associated with HbA1c. Correlations of abundances of the family Ruminococcaceae (A) and the genus Faecalibacterium (B) with HbA1c levels are shown.

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**Fig. 4** – Association between bacterial abundances and numbers of autoantibodies. Correlations between numbers of autoantibodies and HbA1c levels (A), abundances of Bacteroides (B), Streptococcus (C), Ruminococcaceae (D), and Bilophila (E) are shown. Data are presented as mean±SEM.
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Disclosure of interest

The authors declare that they have no conflict of interest.

Authors’ contributions

Huang and Li were responsible for the conception and design of the study and drafting the article. Pei and Wang performed the data analysis and interpretation of data. All authors participated in interpretation of the findings. Hu and Zhang revised it critically for important intellectual content. Guo and Ruan were responsible for acquisition of data. Pan and Fang were responsible for final approval of the version to be submitted.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.diabres.2018.04.032.

REFERENCES


