Liraglutide-induced structural modulation of the gut microbiota in patients with type 2 diabetes mellitus

Junjie Shang[1,*], Fang Liu[2,*], Bing Zhang[1], Kunlun Dong[1], Man Lu[3,4], Rongfeng Jiang[3,4], Yue Xu[3,4], Le Diao[3,4], Jiangman Zhao[3,4] and Hui Tang[3,4]

[1] Nanyang Second General Hospital, Nanyang, Henan Province, China
[2] Kaifeng Central Hospital, Kaifeng, Henan Province, China
[3] Shanghai Biotecan Pharmaceuticals Co., Ltd., Shanghai, China
[4] Shanghai Zhangjiang Institute of Medical Innovation, Shanghai, China
[*] These authors contributed equally to this work.

Corresponding authors
Jiangman Zhao,
zhaojiangman86@163.com
Hui Tang, tang11_23@126.com

ABSTRACT

Accumulating evidence has suggested the importance of gut microbiota in the development of type 2 diabetes mellitus (T2DM). In the present study, 40 patients with T2DM were treated with liraglutide for 4 months. Feces samples and clinical characteristics were collected from these 40 T2DM patients before and after the liraglutide treatment. The diversity and composition of gut microbiota in the two groups were determined by sequencing the V4 region of bacterial 16S rRNA genes. Meanwhile, blood glucose, insulin, hemoglobin A1c (HbA1c), and lipid metabolism were also measured in the pre- and post-liraglutide-treatment groups. We find that Baseline HbA1c was associated with liraglutide treatment response ($R_2 = 0.527$, $\beta = -0.726$, $p < 0.0001$). After adjusted for baseline HbA1c, blood urea nitrogen was associated with liraglutide treatment response. Besides, our results showed reduced gut microbial alpha diversity, different community structure distribution and altered microbial interaction network in patients treated with liraglutide. The liner discriminant analysis (LDA) effect size (LEfSe) analysis showed that 21 species of bacteria were abundant in the pre-liraglutide-treatment group and 15 species were abundant in the post-liraglutide-treatment group. In addition, we also find that *Megamonas* were significantly correlated with older age, diabetes duration and diabetic retinopathy, *Clostridum* were significantly correlated with family history of diabetes and *Oscillospira* were significantly correlated with both diabetic retinopathy and diabetic peripheral neuropathy. Functional analysis based on Kyoto Encyclopedia of Genes and Genomes (KEGG) and cluster of orthologous groups (COG) annotations enriched three KEGG metabolic pathways and six functional COG categories in the post-liraglutide-treatment group. In conclusion, our research suggests that baseline HbA1c, blood urea nitrogen and gut microbiota are associated with the liraglutide treatment applied on patients with T2DM. These findings may contribute to the beneficial effects of liraglutide against diabetes.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a highly prevalent metabolic disorder characterized by the imbalance in blood glucose level and altered lipid profile (*Sharma & Tripathi, 2019*), which is caused by either disturbed insulin secretion, disturbed insulin effect, or usually both (*Petersmann et al., 2019*). The World Health Organization (WHO) has proposed that T2DM could be one of the top ten potent reasons of death worldwide by 2030 (*Kelly et al., 2008*). The increased morbidity and mortality rates related to T2DM are often associated with vascular complications, such as cardiovascular diseases (*Zheng et al., 2018*), nephropathy (*Lu et al., 2017*) and retinopathy (*Maugeri et al., 2018*). Approximately 50% of T2DM patients developed a diabetic peripheral neuropathy (DPN), which is a chronic and progressive disorder affecting the peripheral nervous system (*Edwards et al., 2008*). Diabetic retinopathy (DR) is a microvascular complication of diabetes mellitus and can cause blindness or visual impairment (*Kulkarni et al., 2019*). T2DM is thought to arise due to the overlaps of genetic factors, sedentary lifestyle, poor diet, excessive visceral obesity, and other environmental exposures throughout life (*Salamon et al., 2018*).

In recent years, gut microbiota has been found to play a critical role in the establishment and maintenance of human health. A large number of studies showed that the alteration of microbiota is associated with many human diseases including gastrointestinal diseases (*Tong et al., 2013*), cancer (*Garrett, 2015*), metabolic disease (*Musso, Gambino & Cassader, 2011*), neurodegenerative disorders (*Hsiao et al., 2013*) and cardiovascular (*Wang et al., 2011*). Previous studies of clinical T2DM cases have found compositional changes between the gut microbiome in patients and healthy controls (*Karlsson et al., 2013*). However, the pathophysiological mechanisms that link the microbiota to T2DM have not been well elucidated.

Liraglutide, a glucagon-like peptide 1 (GLP-1) analogue, was approved by the U.S. Food and Drug Administration in 2010 for treating T2DM (*Vilsboll & Garber, 2012*). GLP-1 is an incretin hormone which is secreted by intestinal L cells in response to food ingestion and it shows a significant advance in the treatment of T2DM (*Drucker & Nauck, 2006*). There is evidence that GLP-1 may play a role in the biological function of intestinal epithelium, correlating these effects with changes in gut microbiota (*Hwang et al., 2015*).

However, the effect of liraglutide on gut microbiota in T2DM is not fully understood. Therefore, we performed a randomized study in 40 individuals diagnosed with T2DM, and combined 16S rRNA gene amplicon sequencing to investigate the effects of liraglutide on the composition and function of the gut microbiota.

MATERIALS AND METHODS

Study population

The 40 (including both male and female) T2DM patients were aged 25–83 years, with mean % HemoglobinA1c (HbA1c) concentration of 9.17% (76.67 mmol/mol) (5.3%) (34.43 mmol/mol) − 12.2% (109.84 mmol/mol), and body-mass index (BMI) scores >25 kg/m². HbA1c is the gold standard of glycemic control index and the standardization of HbA1c was considered to be important, which was proceeded by the National Glycohemoglobin

Standardization Program (NGSP) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (*Weykamp, 2013*). All participants had been treated with metformin as a monotherapy (stable doses for 2 months). Relevant exclusion criteria included: taking other glucose-lowering medications and those who had been taking antibiotics in the last 3 months. The recruited participants were randomly assigned to receive 1.2 mg of liraglutide for 4 months. BaselineHbA1c was assessed at before treated-with liraglutide. HbA1c change (liraglutide treatment response) was calculated based on treatment HbA1c value (after 4 months treated-with liraglutide) minus baseline HbA1c value. All 40 T2DM patients were switched from oral metformin to subcutaneous injections of liraglutide for 4 months once daily. The metformin-liraglutide substitution design was applied because a pre-study feasibility assessment reported that it was impossible to recruit sufficient subjects with liraglutide as their primary medication for T2DM (*Hodge et al., 2016*). All procedures were performed in accordance with the ethical standards of the Clinical Research Ethics Committee of the Nanyang Second General Hospital, and written informed consents were obtained from all participants included in the study.

Fecal DNA extraction and 16s rRNA gene sequencing

Total 80 fresh feces samples were collected in sterile collection tubes (Fisher Scientific, Waltham, MA, USA) before and after the 4-month liraglutide treatment and then stored at −20 °C until being transported to the research center in chilled styrofoam containers. They were subsequently stored on-site at −80 °C for further analysis. Microbial DNA was extracted from 200 mg fecal sample using the QIAamp PowerFecal Pro DNA Kit (QIAGEN) which contains a bead-beating step. Briefly, adding 200 mg fecal sample and 800 µl lysis buffer in a beads-containing tube and vortex at maximum speed for 10 min. Take 350 µL supernatant after 1 min centrifugation at 15,000 g and use it in the subsequent steps according to the kit instructions. DNA is finally eluted in 100 µl elution buffer for downstream applications. DNA was amplified using primers targeting the V4 region of the 16S rRNA gene (515F 5′-GTGYCAGCMGCCGCGGTA-3′, 806R 5′-GGACTACNVGGGTWTCTAAT-3′). PCR was run in a Veriti^{TM} 96-Well Thermal Cycler PCR system (Thermo Fisher Scientific) using the following program: 95 °C for 3min, followed by 21 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 5 min. Mixed amplicons were pooled and the sequencing was conducted at Shanghai Biotecan Pharmaceuticals Co., Ltd. (Shanghai, China) using an Illumina Novaseq 6000 Sequencing system (Illumina, USA) according to the manufacturer's instructions.

Sequence data analysis

Sequences were assigned to operational taxonomic units (OTUs) with 97% similarity (Greengenes database: http://greengenes.lbl.gov) into mothur (v.1.39.5), OTU taxonomy was assigned to the Greengenes database for the comparisons, using the Quantitative Insights into Microbial Ecology (QIIME) software package (*Caporaso et al., 2010*). Both Kyoto Encyclopedia of Genes and Genomes (KEGG) (*Langille et al., 2013*) and Clusters of Orthologous Groups of proteins (COG) (*Liu et al., 2019*) pathways were categorized using Phylogenetic Investigation of Communities by Reconstruction of Unobserved

States (PiCRUSt) and both were imported into STAMP (v.2.1.3) for visualization. To identify taxa with differing relative abundances between the two groups, linear discriminant analysis (LDA) effect size (LEfSe) analyses were performed on the website (http://huttenhower.sph.harvard.edu/galaxy). The cut off value was the absolute LDA score (log10) >3.0 with a $p < 0.05$. The alpha diversity "summary.single" script was used to calculate ACE, Chao1, Shannon and Simpson indexs in the mothur software package. "Beta_diversity.py" script was used to calculate the beta diversity in the QIIME software, and the Principal Coordinate Analysis (PCoA) was used to measure beta diversity in each group. Coabundance network analysis was based on Spearman correlation. Only genera present in at least 60% of samples were used for correlation analysis, and only connections with a $r > 0.6$ or < -0.6 and $P < 0.05$ were used for network building on the basis of Igraph R package and imported into Cytoscape (v.3.6.0) for visualization.

Statistical analysis

The data are shown as the mean ± standard error of the mean (SEM). Bacterial taxa that were differentially abundant were identified by using the Wilcoxon rank-sum test (for two groups) or Kruskal-Wallis test (for more than two groups) in R studio (v.3.6.1). Clinical data analyses between the two groups were conducted by Wilcoxon signed–rank test or t-test using Prism version 6.0 (GraphPad, San Diego, CA, USA). Linear regression analysis was performed using R studio (v.3.6.1). A $p < 0.05$ was considered statistically significant.

RESULTS

Baseline HbA1c is a key predictor of liraglutide treatment response

We recruited 40 individuals with T2DM to receive 1.2 mg liraglutide for 4 months. Clinical characteristics of the 40 individuals before and after the 4-month liraglutide treatment (termed L0 and L4, respectively) are presented in Table S1. As baseline HbA1c is a major predictor in blood glucose therapies, we assessed the relationship between baseline HbA1c and HbA1c change using linear regression analysis. The result indicated that baseline HbA1c explains 52.7% of liraglutide treatment response variation ($R^2 = 0.527$, $\beta = -0.726$, $P < 0.0001$, Fig. 1). In addition, BMI, HbA1c, homeostasis model assessment of insulin resistance (HOMA-IR), fasting blood glucose, 2-hour postprandial blood glucose, total cholesterol, triglycerides, HDL-C, and LDL-C were significantly lower in the post-liraglutide-treatment group than the pre-liraglutide-treatment groupusing Wilcoxon signed–rank test or t-test . However, there were no notable differences observed in fasting insulin, serum creatinine and the blood urea nitrogen between the two groups (Table S2).

Furthermore, due to many clinical characteristics may be associated with baseline HbA1c, adjusted for baseline HbA1c was suggested to avoid erroneous treatment response. So we used multiple linear regression to analyze the association between the baseline HbA1c and the baseline covariates (11 clinical characteristics), and the association between the baseline covariates and liraglutide treatment response after with or without adjustment for baseline HbA1c. However, the results indicated that none clinical characteristics were associated with both baseline HbA1c and liraglutide treatment response after without adjustment for
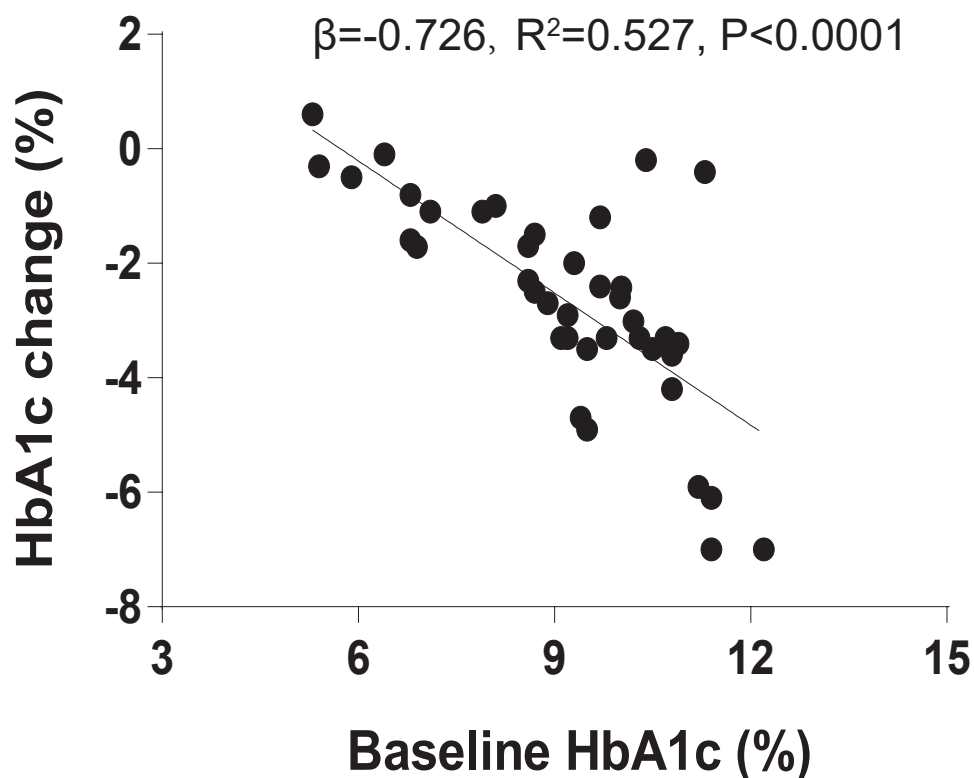
Figure 1 **Baseline HbA1c is associated with liraglutide treatment response.** The association between % baseline HbA1c and % HbA1c change using linear regression analysis ($R^2 = 0.527$, $\beta = -0.726$, $P < 0.0001$).

baseline HbA1c. Only the blood urea nitrogen was associated with liraglutide treatment response after adjustment for baseline HbA1c (Table S3).

Characteristics of the pyrosequencing results

To characterize the effects of liraglutide on the gut microbiome, we performed high-throughput sequencing of the V4 regions of 16S rRNA genes from the 80 fecal samples collected from the two groups. A total of 8,822,088 high-quality sequences and 7488 OTUs (97% similarity) were obtained from the 80 samples, and the average number of reads was 100, 626 and OTUs was 296 for each individual (Table S4). The reads/OTUs were assigned to 34 different phyla, and the top 3 dominant bacterial phyla of the two groups were Firmicutes, Bacteroidetes and Proteobacteria.

Liraglutide modifies gut microbiota alpha and beta diversity in the T2DM

Since the participants have different duration of T2DM, we divided the 40 T2DM patients into three diabetes duration groups (short-duration group (<5 years); medium- duration group (5-10 years) and long-duration group (≥10 years)) and analyzed alpha and beta diversity in gut microbiota of the three groups. Alpha diversity is an index reflecting the variety of microbial species in stool samples and we also examined the patterns of beta

diversity by calculating the dissimilarity in the composition of gut microbial (*Rapacciuolo et al., 2019*). The results indicated that the three duration groups had no significant difference in gut microbiota alpha and beta diversity (Figs. S1 and S2). Therefore, being treated with metformin for 2 months do not alter the fecal microbiome in T2DM patients. In order to evaluate the effect of liraglutide treatment on gut microbiota diversity, we analyzed the 80 fecal samples collected from pre- and post-liraglutide-treatment groups. The alpha diversity indices (the ACE, Chao1, Shannon and Simpson index) were used to describe alpha diversity. The community richness (ACE and Chao1) was significantly different between the two groups (Figs. 2A and 2B), showing a lower abundance in the post-liraglutide-treated group. However, there were no significant difference in the community diversity (Shannon and Simpson) of the two groups (Figs. S3A and S3B). As to the beta diversity, a principal-component analysis (PCoA) of a nonmetric multidimensional scaling plot (on a Bray-Curtis distance matrix) and unweighted UniFrac distances revealed significant qualitative differences in gut microbial community structure between the two groups (Figs. 2C and 2D), but there was no difference in weighted UniFrac distances (Fig. S3C).

Liraglutide alters gut microbiota composition

To assess the effects of liraglutide on gut microbiota composition, a taxonomy-based comparison was performed and a broad overview of our taxonomic data from the 80 samples was given in Fig. 3A. A heat map was constructed to visualize the top 30 genus in the two groups (Fig. 3A). We also conducted a coabundance network analysis to investigate how different gut bacteria could interact with each other. The results indicated that 4 months of liraglutide treatment promoted an increased number of positive correlations among microbial genera, especially those within Firmicutes and Bacteroidetes. Negative correlation was identified in *Ruminococcus* (Firmicutes) and *Actinomyces* (Actinobacteria) (Fig. 3B).

Phylogenetic and taxonomic profiles of gut microbiota

To analyze the statistical differences in microbial communities between the pre- and post-liraglutide-treatment of T2DM, the significant differences based on genomic characteristics were further confirmed by LEfSe analysis, which used LDA coupled with effect size measurement to identify bacterial taxa with sequences that differed in the abundance of the two groups.

Cladograms were obtained by the LEfSe analysis, which showed the most significantly difference at taxonomic levels between the two groups. The size of each circle represents the abundance of certain bacteria (Fig. 4A).

Upon analyzing the fecal samples, 36 phylotypes were identified as high-dimensional biomarkers (Table S5). Twenty-one species of bacteria were abundant in the pre-liraglutide-treatment group and 15 species were abundant in the post-liraglutide-treatment group. At the phylum level, the abundance of Fusobacteria was significantly higher in the pre-liraglutide-treatment group, while Verrucomicrobia and Actinobacteria were significantly higher in the post-liraglutide-treatment group. At the genus level, nine genera were found in significantly high abundances in the pre-liraglutide-treatment group. These
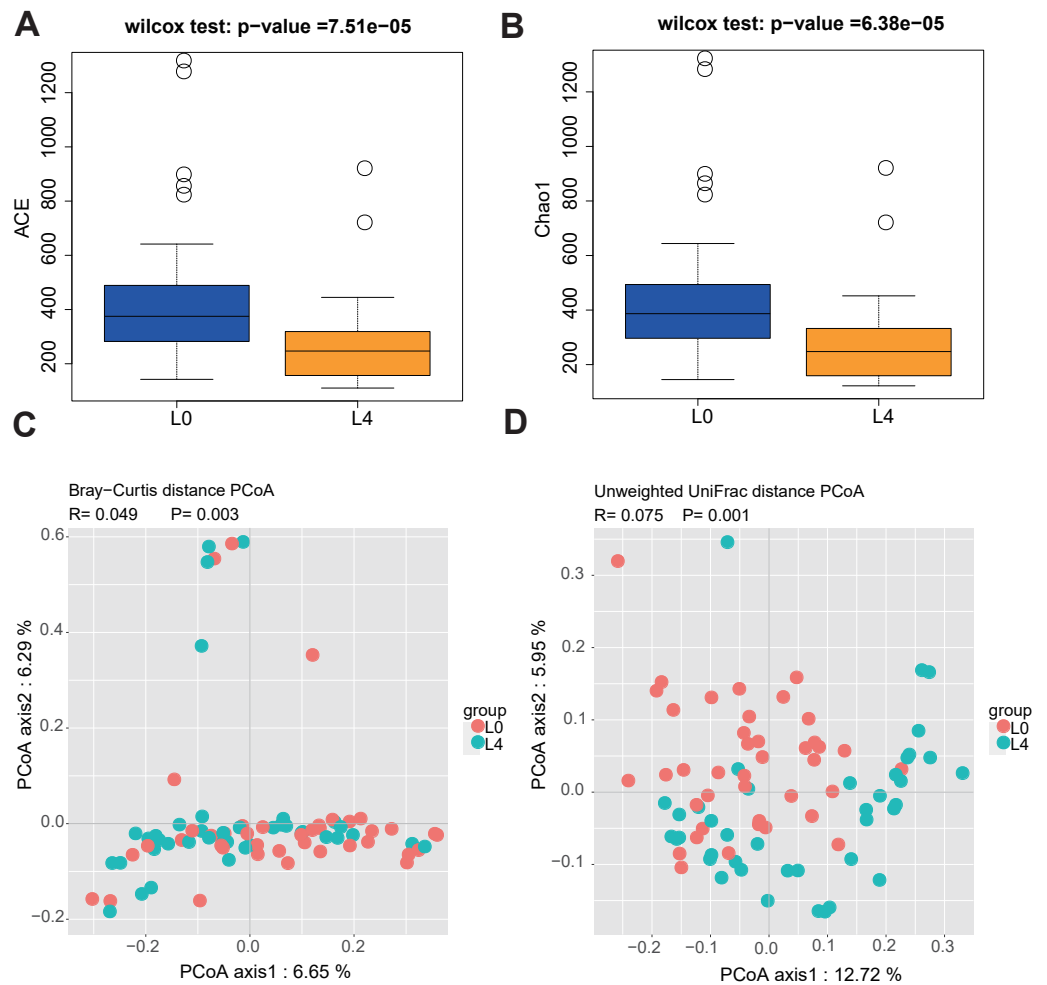
Figure 2 **Liraglutide treatment affected the microbiota alpha diversity and beta diversity.** (A–B) ACE and Chao1 were used to analyze the alpha diversity. Statistic analysis was performed by Wilcoxon rank-sum test, $*P < 0.05$. (C–D) PCoA of fecal microbiota from the two groups of patients using a Bray-Curtis distance matrix (C) and unweighted UniFrac distances (D). Statistical analysis was performed by ANOSIM test, $*P < 0.05$.

genera included *Acinetobacter* ($p = 0.016$), *Oscillospira* ($p = 0.013$), *Acidaminococcus* ($p = 0.021$), *Succinatimonas* ($p = 0.042$), *S24_7* ($p = 0.008$), *Megamonas* ($p = 0.0005$), *Alistipes* ($p = 0.035$), *Fusobacterium* ($p = 0.017$) and *Megasphaera* ($p = 0.0002$). The genera *Collinsella* ($p = 0.011$), *Akkermansia* ($p = 0.002$) and *Clostridium* ($p = 0.002$) were enriched in the post-liraglutide-treatment group (Fig. 4B).

Correlation between clinical features and gut microbiota

According to the LDA analysis (LDA value > 3.0), we discovered the relative abundance of 12 bacterial genera (Table S5) between the two groups. To investigate the inter associations between gut microbiota and clinical status of the host, we identified the statistical correlations between the 12 genera and the 11 clinical features, including age, sex, BMI, family history of diabetes, diabetes duration, family history of cardiovascular disease,
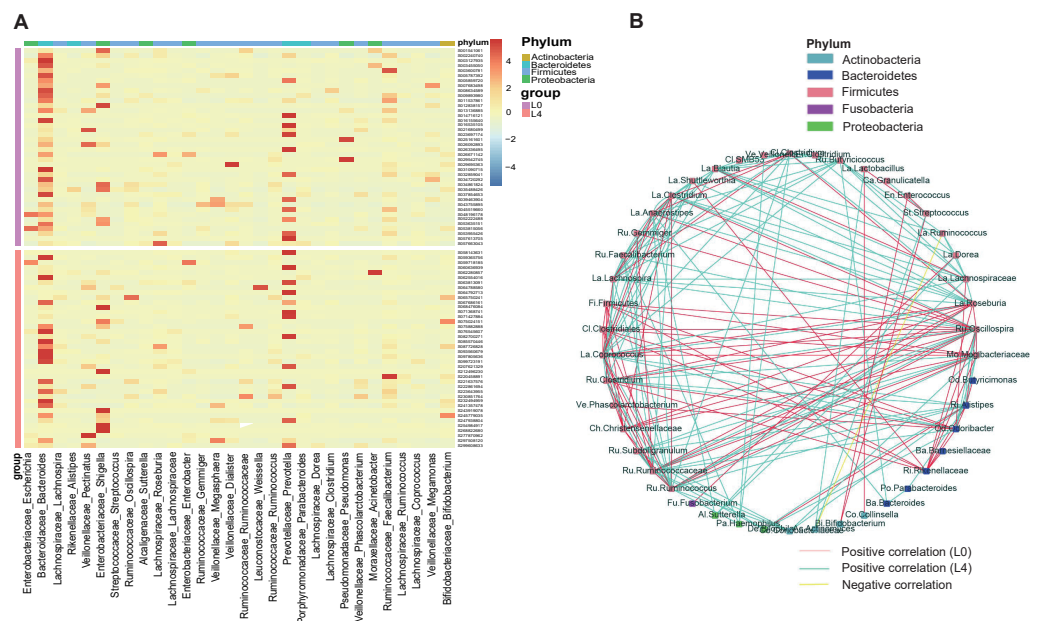
Figure 3 **Liraglutide treatment promoted rapid changes in the composition of the gut microbiota.**
(A) Heat map showing changes in the abundance of bacterial strains before (L0) and after the 4-month li-
raglutide treatment (L4) (only the top 30 genera are listed here). (B) Genus–genus coabundance network
of the pre- (L0) and the post-liraglutide-treatment group (L4). The edges indicate Spearman correlations
of $r > 0.5$ or $r < -0.5$ and $*P < 0.05$ between the genera present in at least 60% samples.

family history of diabetic nephropathy (DN), family history of diabetic retinopathy (DR),
family history of diabetic peripheral neuropath (DPN), smoking status and alcoholism
status (Table S6).

First, we found that the abundance of *Megamonas* was significantly lower in the older
age group (≥ 50) ($p = 0.014$), medium- and long-duration groups ($p = 0.006$) (Figs. 5A
and 5B). What's more, the levels of *Clostridum* genus was lower in the family history of
diabetes ($p = 0.043$) group (Fig. 5C). *Oscillospira was* significantly different between the
Non-DR group and DR groups, in which severe non-proliferative DR (NPDR) sub-group
had the highest abundance of *Oscillospira* ($p = 0.038$) (Fig. 5D). We also found that the
abundance of *Megamonas* was lower in the DR group ($p = 0.005$) (Fig. 5E). Last but not
least, the higher abundance of *Oscillospira* was found in the DPN group, but not in the
non-DPN group ($p = 0.040$) (Fig. 5F).

Effect of liraglutide-treatment administration on metabolic pathways
KEGG and COG pathway comparisons were performed to explore potential differences
in the functional composition of the microbiome between the pre- and post-liraglutide-
treatment group. Figure 6 highlights the significant differences in the distribution of
metabolic pathways, and compares the microbiota functions between the two groups. As
listed in Fig. 6A, seven differences were observed in KEGG pathways. Three KEGG pathways
(including G protein-coupled receptors, Glycolysis/Gluconeogenesis and Selenocompound
metabolism) were enriched in the post-liraglutide-treatment group, and 4 KEGG
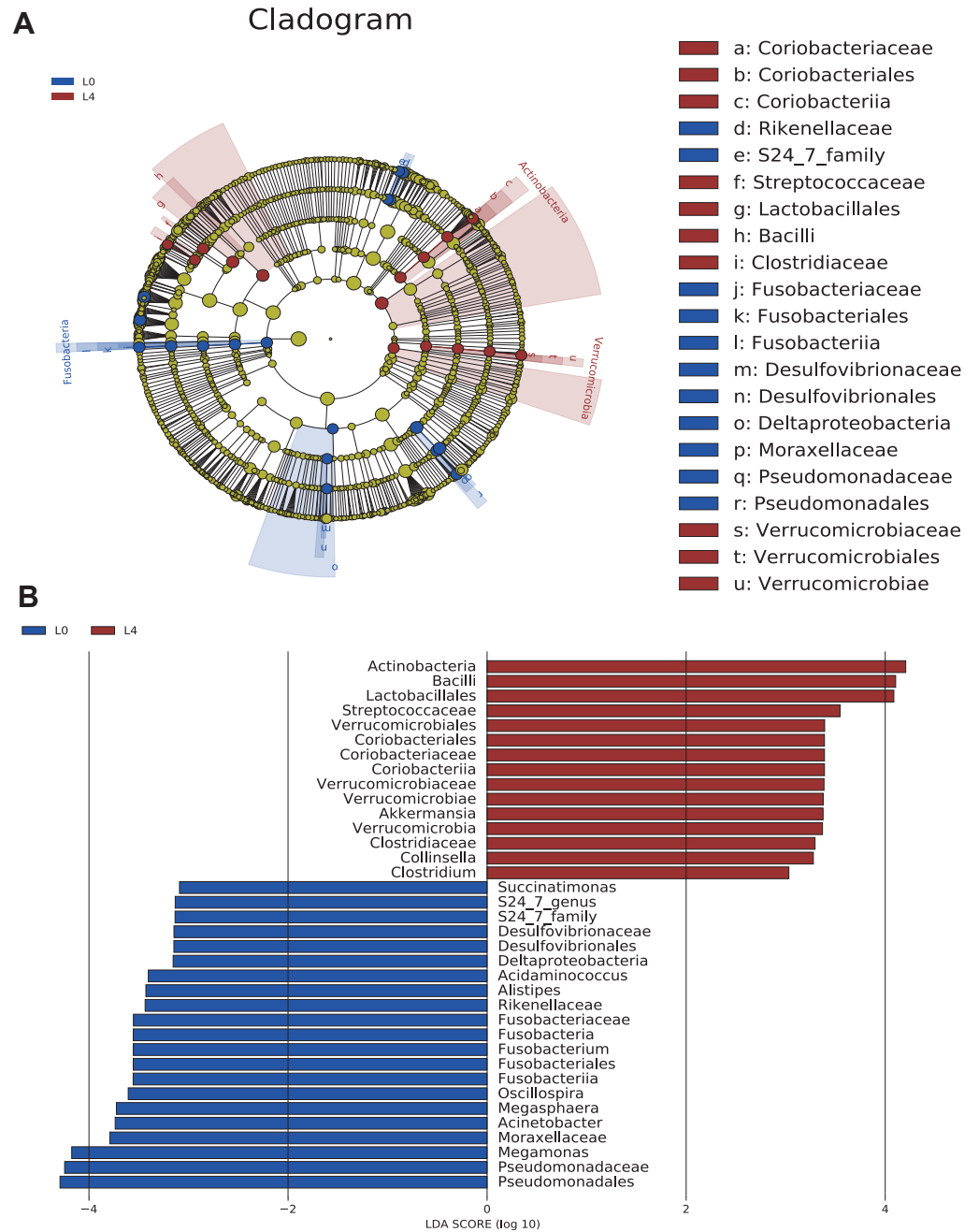
Figure 4 **The overall structure and composition of the gut microbiota difference between the pre- and post-liraglutide-treatment group.** (A) The cladogram depicts the phylogenetic distribution of microbial lineages in fecal samples taken before (L0) and after the 4-month liraglutide treatment (L4). Differences in abundant microbiota are listed and distinguished by different color (blue for L0 group and brown for L4 group). (B) Key phylotypes of the gut microbiota responding to liraglutide treatment. The histogram shows the lineages with LDA values >3.0 and with a *P < 0.05 as determined by LEfSe analysis.
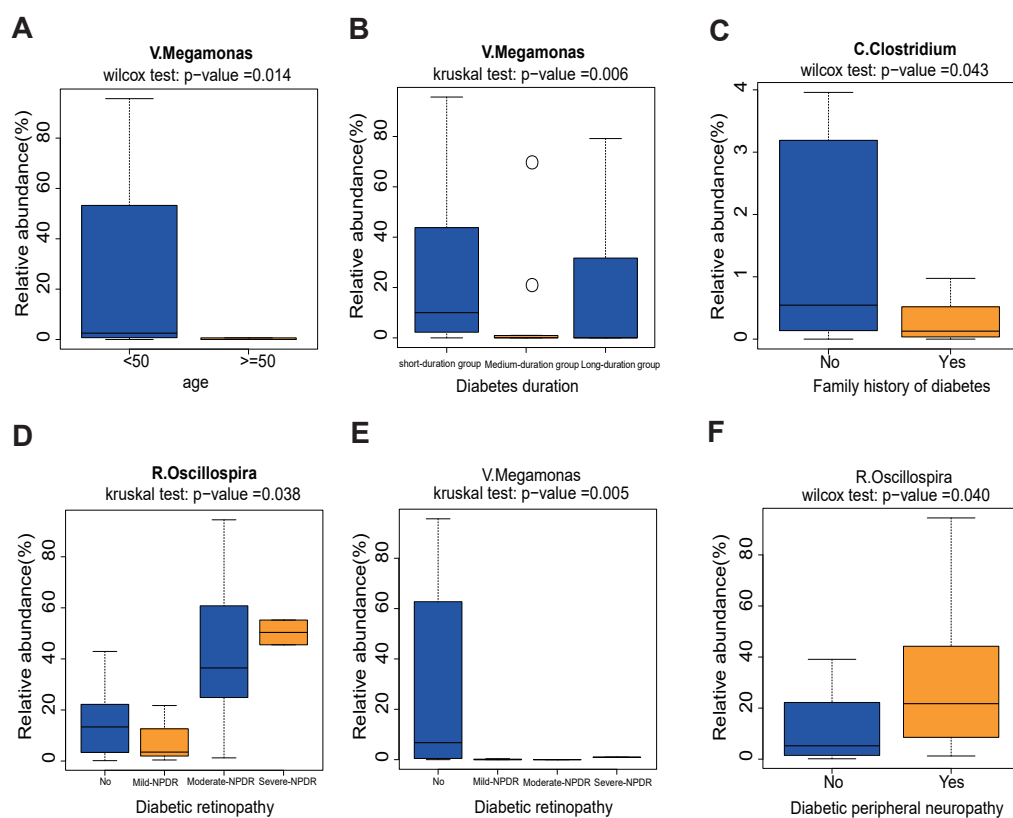
Figure 5 **Correlation analysis between 11 clinical features and 12 genera.** (A) Comparison of 12 relative abundance genera between <50 age and ≥50 age group. Obviously, *Megamonas* was lower in the ≥50 age group. (B) The abundance of *Megamonas* was lower in the medium- and long-duration groups. (C) The abundance of *Clostridium* was lower in the family history of diabetes group. (D–E) The abundance of *Oscillospira* was higher while the abundance of *Megamonas* was lower in the DR group. (F) The abundance of *Oscillospira* was higher in the DPN group. Statistical analysis was performed by Wilcoxon rank-sum test or Kruskal-Wallis test, ∗*P* < 0.05.

pathways (including tropane, piperidine and pyridine alkaloid biosynthesis, phenylalanine metabolism, porphyrin and chlorophyll metabolism, and styrene degradation) were enriched in the pre-liraglutide-treatment group.

As shown in Fig. 6B, 17 different functional COG pathways between the two groups were found. Six of them were enriched in the post-liraglutide-treatment group, including predicted ABC-type transport system involved in lysophospholipase L1 biosynthesis, acetate kinase, phosphatidylglycerophosphate synthase, predicted choline kinase involved in lipopolysaccharides (LPS) biosynthesis, predicted glutamine amidotransferase and putative cell wall-binding domain. In contrast, the pre-liraglutide-treatment group were enriched in ABC-type Co2+ transport system, periplasmic component, ABC-type Fe3+ transport system, periplasmic component, transcriptional regulator containing PAS, AAA-type ATPase, and DNA-binding domains, acetyl-CoA hydrolase, N-acyl-D-aspartate/D-glutamate deacylase, Na+/proline symporter, permeases of the drug/metabolite transporter
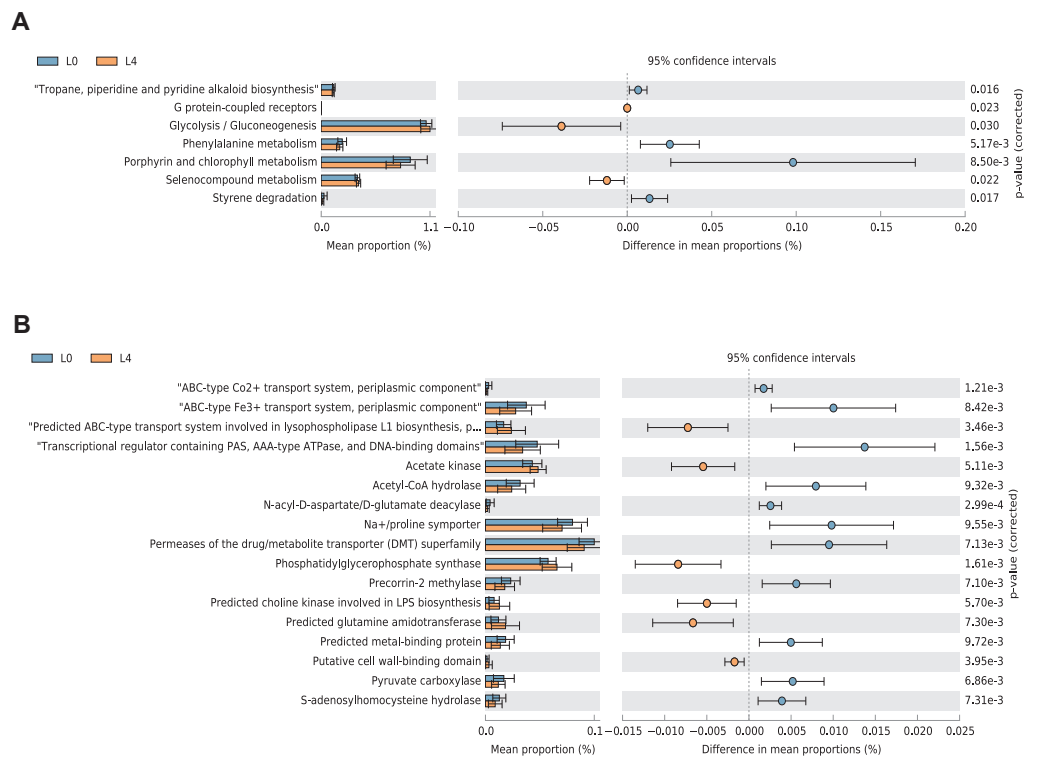
Figure 6 Differential PICRUSt-predicted KEGG and COG pathways in individuals before (L0) and after the 4-months liraglutide treatment (L4). The KEGG (A) and COG (B) pathways demonstrating seven and 17 significant differences between the two groups, respectively. For each comparison in predicted KEGG or COG pathways, the mean proportion and the 95% CI difference in mean proportions are also illustrated. Statistical analysis was performed by Welch's t-test, $*P < 0.05$.

(DMT) superfamily, precorrin-2 methylase, predicted metal-binding protein, pyruvate carboxylase and s-adenosylhomocysteine hydrolase.

DISCUSSION

T2DM is a complex metabolic disorder and public health issue throughout the world. It is also one of the most severe chronic diseases that affect human health. According to the estimation, the prevalence of diabetes may increase 69% in developing countries and 20% in developed countries between 2010 and 2030 (*Shaw, Sicree & Zimmet, 2010*). The metabolic potential of the gastrointestinal tract and its microbiota are increasingly recognized as promising targets to improve T2DM treatment (*Brunkwall & Orho-Melander, 2017*). Thus, there is an urgent need in the development of more efficient prevention and treatment strategies.

As a GLP-1 analogue, liraglutide has shown the therapeutic effects by changing the structural of gut microbiota in diabetic male rats (*Zhang et al., 2018*). In this study, although all participants were stable on metformin as a monotherapy for 2 months before the recruitment, neither alpha nor beta diversity was different among the three diabetes duration groups. Then all 40 T2DM patients were switched from oral metformin

to subcutaneous injections of liraglutide for 4 months. We found that the liraglutide treatment lowered BMI, blood glucose, HbA1c, HOMA-IR and relieved dyslipidemia in T2DM patients. Similarity, in a diabetic rat study, insulin level and HOMA-IR were significantly decreased (*Zhang et al., 2018*), and blood glucose and body weight were much lower after being treated with liraglutide (*Wang et al., 2016*). In the present study, we found that baseline HbA1c is associated with liraglutide treatment response. As clinical characteristics may be associated with baseline HbA1c, many T2DM studies have adjusted for baseline HbA1c to avoid incorrect treatment response using linear regression method (*Jones et al., 2016*; *Thong et al., 2014*). In this study, neither baseline HbA1c nor liraglutide treatment response after without adjustment for baseline HbA1c was associated with baseline covariates. However, the blood urea nitrogen was associated with treatment response after adjustment for baseline HbA1c. Many studies have indicated that blood urea nitrogen is associated with increased an risk of insulin use in diabetes mellitus (*Xie et al., 2018a*; *Xie et al., 2018b*). Jones et al. reported that false positive associations may arise and true associations may be covered up by negative results if without adjustment for baseline HbA1c (*Jones et al., 2016*). In this study, without adjustment for baseline HbA1c, the blood urea nitrogen was not associated with treatment response; while adjustment for baseline HbA1c, the blood urea nitrogen was associated with treatment response. Furthermore, we analyzed the effects of liraglutide on the gut microbiota of the 80 samples collected from the pre- and post-liraglutide-treatment groups. Several interesting results were observed. First of all, at the phyla level, Firmicutes, Bacteroidetes and Proteobacteria were the top 3 dominant bacterial phyla between the two groups. A previous study reported that Bacteroidetes and Proteobacteria, could produce LPS, and subsequently trigger inflammatory response and contribute to the development of diabetes (*Larsen et al., 2010*). The diabetic rat microbiome was profoundly dominated by the phyla Firmicutes and Bacteroides, while liraglutide-treated rat group had a reduced Firmicutes/Bacteroidetes ratio and relative abundance of Tenericutes (*Zhang et al., 2018*). According to our taxonomic data from the 80 samples, patients who had enriched genus *Bacteroides* usually had low abundance of genus *Prevotella*, which is consistent with the previous study performed in colorectal cancer (*Yachida et al., 2019*). Zhang et al. reported that *Prevotella* genera was much more lower in liraglutide-treated rats (*Zhang et al., 2018*). Secondly, by using a Bray-Curtis distance matrix and unweighted UniFrac PCoA analysis, we confirmed that two major components in the gut microbiota of the two groups were structurally separated from each other. What's more, according to LEfSe analysis, 9 genera including *Acinetobacter*, *Oscillospira*, *Acidaminococcus*, *Succinatimonas*, *S24_7*, *Megamonas*, *Alistipes*, *Fusobacterium* and *Megasphaera* were found in significantly high abundances in the pre-liraglutide-treatment group. In a previous study, Li et al. reported that *Acinetobacter* was significantly abundant in T2DM group than that in healthy subjects (*Li et al., 2019*). *Oscillospira* was reported to be significantly abundant in high-fat diet mice group than in a chow diet mice group (*Wu et al., 2019*). However, *Fusobacterium* genera in fecal samples were not significantly different between the T2DM and healthy people (*Sedighi et al., 2017*). Therefore, large studies were needed to verify this issue in near future. According to our research, *Megamonas* was significantly lower in the older group, medium- and long-

duration group, which is in accord with the study that *Megamonas* was significant lower in longevity village communities than urbanized town communities (*Park et al., 2015*). A similar study indicated that the abundance of *Alistipes* was significantly increased in rat of T2DM (*Zhu et al., 2020*). *Clostridum* was lower in the T2DM family history of diabetes group, similarly, *Clostridum* was significantly lower in the fecal samples of diabetic patients than in those of control subjects in Japanese (*Yamashiro, 2017*). Another study further indicated that *Clostridium* was negatively correlated with fasting blood glucose, HbA1c and insulin levels (*Karlsson et al., 2013*).

On contrary, liraglutide-treated group had an increased level of *Collinsella*, *Akkermansia* and *Clostridium* at the genus level. In a pre-clinical study, the abundance of *Akkermansia* was increased by the metformin treatment in the mice fed by high fat diet (HFD), and oral administration of *Akkermansia* enhanced glucose tolerance and attenuated adipose tissue inflammation in HFD-fed mice (*Shin et al., 2014*). Another study reported the low abundance of *Clostridium* in European women with T2DM, as well as the negative correlation of *Clostridium* with fasting blood sugar, glycated hemoglobin, insulin, and plasma triglycerides (*Karlsson et al., 2013*). In our study, after being treated with liraglutide for 4 months, the abundance of *Clostridium* was decreased in the family history of diabetes group, which is consistent with the findings that the abundance of *Clostridium* is lower in diabetic patients (*Yamashiro, 2017*). In the diabetic rat liraglutide-treated study, LEfSe analysis showed 11 bacteria significantly difference between the liraglutide-treated group and diabetic group. The genera Flavonifractor and Lachnoclostridium, species Ruminococcus_gnavus, Flavonifractor_plautii, and Bacteroides_acidifaciens were much more abundant in the liraglutide-treated group than the diabetic group. The reduced bacteria were mainly in the family Christensenellaceae, genera Christensenellaceae_R_7_group, the genera Ruminococcaceae_UCG_010, Ruminoclostridium_6, and Prevotella_9, and the class Mollicutes (*Zhang et al., 2018*). These results indicated that microbiome structures of rat and human were mainly different.

Finally yet importantly, the PICRUSt analysis revealed 7 different KEGG pathways and 17 COG pathways. KEGG analysis further indicated that these gut bacteria in T2DM were strongly associated with the dysregulation of several metabolic processes such as glycolysis/gluconeogenesis metabolism; phenylalanine metabolism; porphyrin and chlorophyll metabolism; and selenocompound metabolism. For example, phenylalanine metabolism, showed lower abundance in the post-liraglutide-treatment group. It is reported that phenylalanine was associated with insulin resistance, and may increase the risk of T2DM (*Guasch-Ferre et al., 2016*). Glycolysis/gluconeogenesis pathway was higher in the post-liraglutide-treatment group, which is consistent with the findings that glycolysis/gluconeogenesis related metabolites is significantly associated with T2DM risk in a Mediterranean population (*Guasch-Ferre et al., 2020*). In addition, we found 17 different functional COGs between the two groups. For example, periplasmic component, ATPase component and permeases of the drug/metabolite transporter (DMT) were enriched in the pre-liraglutide-treatment group, which promote glucose and ribose/galactoside using to regulate energy according to the previous study (*Liu et al., 2019*). Considering the fact that T2DM is a metabolic disease and several metabolic disorder phenotypes were observed in

T2DM patients, we speculated that gut microbiota affects the host via metabolites, which provides a further understanding between the microbiome–metabolome interaction in T2DM and may be helpful for the diagnosis and treatment of T2DM in the future.

There were still several limitations in this study. Specifically, the sample size was relatively small to represent the combined effects of T2DM and liraglutide. Besides, we did not recruit a 4-month-metformin-treated group to compare with the current- liraglutide-treated group. Although our collected clinical characteristics were significant changes between the pre- and post-liraglutide treatment groups. While adjustment for baseline HbA1c, only blood urea nitrogen was associated with liraglutide treatment response in the 40 T2DM patients. Therefore, it is urgent to enlarge cohort to verify this issue in the future. Due to the extremely complicated interactions between gut microbiota and host, as well as our knowledge and research capability, the potential mechanisms behind this interaction were not further explored. Meanwhile, metagenomic sequencing has been widely employed to explore novel changes in the functional potential of the microbiota. However, in the present study, the results of prediction COG and KEGG functional analysis were analyzed by the method of 16S rRNA gene sequencing. Besides, due to the small sample size, we only covered part of the correlations between gut microbiota and clinical features. Therefore, to fully elucidate the modifiable capacity of the gut microbiota and its potential applications in the prevention and treatment for type 2 diabetes, large longitudinal, interventional studies and multicenter strategies are further required.

In conclusion, our findings suggest that liraglutide treatment is associated with baseline HbA1c, blood urea nitrogen and gut microbiota composition in T2DM patients. This may contribute to the beneficial effects of liraglutide against diabetes. In particular, the phenylalanine metabolism, porphyrin and chlorophyll metabolism and selenocompound metabolism pathways were predicted by KEGG pathways without no functional analysis in T2DM yet. So further researches are required to elucidate the mechanisms by which liraglutide affects the gut microbiota of diabetes patients, and this may serve as potential therapeutic targets for T2DM.

Funding

This work was supported by the Key Technologies R & D Project of Nanyang Science and Technology Bureau (Henan Provincial Medical Science and Technique Program, 2018020989- KJGG2018101). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Key Technologies R & D Project of Nanyang Science and Technology Bureau: 2018020989-KJGG2018101.

Competing Interests

Man Lu, Rongfeng Jiang, Yue Xu, Le Diao, Jiangman Zhao and Hui Tang are employed by Shanghai Biotecan Pharmaceuticals Co., Ltd.

The authors declare there are no competing interests.

Author Contributions

- Junjie Shang and Fang Liu conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Bing Zhang and Kunlun Dong performed the experiments, prepared figures and/or tables, and approved the final draft.
- Man Lu and Rongfeng Jiang analyzed the data, prepared figures and/or tables, and approved the final draft.
- Yue Xu performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Le Diao performed the experiments, prepared figures and/or tables, and approved the final draft.
- Jiangman Zhao analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Hui Tang conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Human Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The Medical Ethics Committee of Nanyang Second People's Hospital approved this study.

Data Availability

Data are available at the NCBI BioProject database: PRJNA665123.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.11128#supplemental-information.

REFERENCES

Brunkwall L, Orho-Melander M. 2017. The gut microbiome as a target for prevention and treatment of hyperglycaemia in type 2 diabetes: from current human evidence to future possibilities. *Diabetologia* **60**:943–951 DOI 10.1007/s00125-017-4278-3.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight

R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**:335–336 DOI 10.1038/nmeth.f.303.

Drucker DJ, Nauck MA. 2006. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* **368**:1696–1705 DOI 10.1016/S0140-6736(06)69705-5.

Edwards JL, Vincent AM, Cheng HT, Feldman EL. 2008. Diabetic neuropathy: mechanisms to management. *Pharmacology and Therapeutics* **120**:1–34 DOI 10.1016/j.pharmthera.2008.05.005.

Garrett WS. 2015. Cancer and the microbiota. *Science* **348**:80–86 DOI 10.1126/science.aaa4972.

Guasch-Ferre M, Hruby A, Toledo E, Clish CB, Martinez-Gonzalez MA, Salas-Salvado J, Hu FB. 2016. Metabolomics in prediabetes and diabetes: a systematic review and meta-analysis. *Diabetes Care* **39**:833–846 DOI 10.2337/dc15-2251.

Guasch-Ferre M, Santos JL, Martinez-Gonzalez MA, Clish CB, Razquin C, Wang D, Liang L, Li J, Dennis C, Corella D, Munoz-Bravo C, Romaguera D, Estruch R, Santos-Lozano JM, Castaner O, Alonso-Gomez A, Serra-Majem L, Ros E, Canudas S, Asensio EM, Fito M, Pierce K, Martinez JA, Salas-Salvado J, Toledo E, Hu FB, Ruiz-Canela M. 2020. Glycolysis/gluconeogenesis- and tricarboxylic acid cycle-related metabolites, Mediterranean diet, and type 2 diabetes. *American Journal of Clinical Nutrition* **111**:835–844 DOI 10.1093/ajcn/nqaa016.

Hodge RJ, Paulik MA, Walker A, Boucheron JA, McMullen SL, Gillmor DS, Nunez DJ. 2016. Weight and glucose reduction observed with a combination of nutritional agents in rodent models does not translate to humans in a randomized clinical trial with healthy volunteers and subjects with type 2 diabetes. *PLOS ONE* **11**:e0153151 DOI 10.1371/journal.pone.0153151.

Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, Codelli JA, Chow J, Reisman SE, Petrosino JF, Patterson PH, Mazmanian SK. 2013. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* **155**:1451–1463 DOI 10.1016/j.cell.2013.11.024.

Hwang I, Park YJ, Kim YR, Kim YN, Ka S, Lee HY, Seong JK, Seok YJ, Kim JB. 2015. Alteration of gut microbiota by vancomycin and bacitracin improves insulin resistance via glucagon-like peptide 1 in diet-induced obesity. *FASEB Journal* **29**:2397–2411 DOI 10.1096/fj.14-265983.

Jones AG, Lonergan M, Henley WE, Pearson ER, Hattersley AT, Shields BM. 2016. Should studies of diabetes treatment stratification correct for baseline HbA1c? *PLOS ONE* **11**:e0152428 DOI 10.1371/journal.pone.0152428.

Karlsson FH, Tremaroli V, Nookaew I, Bergstrom G, Behre CJ, Fagerberg B, Nielsen J, Backhed F. 2013. Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* **498**:99–103 DOI 10.1038/nature12198.

Kelly T, Yang W, Chen CS, Reynolds K, He J. 2008. Global burden of obesity in 2005 and projections to 2030. *International Journal of Obesity* **32**:1431–1437 DOI 10.1038/ijo.2008.102.

Kulkarni S, Kondalkar S, Mactaggart I, Shamanna BR, Lodhi A, Mendke R, Kharat J, Kapse R, Dole K, Deshpande M. 2019. Estimating the magnitude of diabetes mellitus and diabetic retinopathy in an older age urban population in Pune, western India. *BMJ Open Ophthalmology* **4**:e000201 DOI 10.1136/bmjophth-2018-000201.

Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology* **31**:814–821 DOI 10.1038/nbt.2676.

Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, Al-Soud WA, Sorensen SJ, Hansen LH, Jakobsen M. 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLOS ONE* **5**:e9085 DOI 10.1371/journal.pone.0009085.

Li S, Yi G, Peng H, Li Z, Chen S, Zhong H, Chen Y, Wang Z, Deng Q, Fu M. 2019. How ocular surface microbiota debuts in type 2 diabetes mellitus. *Frontiers in Cellular and Infection Microbiology* **9**:202 DOI 10.3389/fcimb.2019.00202.

Liu F, Li J, Guan Y, Lou Y, Chen H, Xu M, Deng D, Chen J, Ni B, Zhao L, Li H, Sang H, Cai X. 2019. Dysbiosis of the gut microbiome is associated with tumor biomarkers in lung cancer. *International Journal of Biological Sciences* **15**:2381–2392 DOI 10.7150/ijbs.35980.

Lu M, Yin N, Liu W, Cui X, Chen S, Wang E. 2017. Curcumin ameliorates diabetic nephropathy by suppressing NLRP3 inflammasome signaling. *BioMed Research International* **2017**:1516985 DOI 10.1155/2017/1516985.

Maugeri A, Mazzone MG, Giuliano F, Vinciguerra M, Basile G, Barchitta M, Agodi A. 2018. Curcumin modulates DNA methyltransferase functions in a cellular model of diabetic retinopathy. *Oxidative Medicine and Cellular Longevity* **2018**:5407482 DOI 10.1155/2018/5407482.

Musso G, Gambino R, Cassader M. 2011. Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. *Annual Review of Medicine* **62**:361–380 DOI 10.1146/annurev-med-012510-175505.

Park SH, Kim KA, Ahn YT, Jeong JJ, Huh CS, Kim DH. 2015. Comparative analysis of gut microbiota in elderly people of urbanized towns and longevity villages. *BMC Microbiology* **15**:49 DOI 10.1186/s12866-015-0386-8.

Petersmann A, Muller-Wieland D, Muller UA, Landgraf R, Nauck M, Freckmann G, Heinemann L, Schleicher E. 2019. Definition, classification and diagnosis of diabetes mellitus. *Experimental and Clinical Endocrinology and Diabetes* **127**:S1–S7 DOI 10.1055/a-1018-9078.

Rapacciuolo G, Beman JM, Schiebelhut LM, Dawson MN. 2019. Microbes and macro-invertebrates show parallel beta-diversity but contrasting alpha-diversity patterns in a marine natural experiment. *Proceedings of the Royal Society B: Biological Sciences* **286**:20190999 DOI 10.1098/rspb.2019.0999.

Salamon D, Sroka-Oleksiak A, Kapusta P, Szopa M, Mrozinska S, Ludwig-Slomczynska AH, Wolkow PP, Bulanda M, Klupa T, Malecki MT, Gosiewski T. 2018. Characteristics of gut microbiota in adult patients with type 1 and type 2 diabetes based on nextgeneration sequencing of the 16S rRNA gene fragment. *Polish Archives of Internal Medicine* **128**:336–343 DOI 10.20452/pamw.4246.

Sedighi M, Razavi S, Navab-Moghadam F, Khamseh ME, Alaei-Shahmiri F, Mehrtash A, Amirmozafari N. 2017. Comparison of gut microbiota in adult patients with type 2 diabetes and healthy individuals. *Microbial Pathogenesis* **111**:362–369 DOI 10.1016/j.micpath.2017.08.038.

Sharma S, Tripathi P. 2019. Gut microbiome and type 2 diabetes: where we are and where to go? *Journal of Nutritional Biochemistry* **63**:101–108 DOI 10.1016/j.jnutbio.2018.10.003.

Shaw JE, Sicree RA, Zimmet PZ. 2010. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice* **87**:4–14 DOI 10.1016/j.diabres.2009.10.007.

Shin NR, Lee JC, Lee HY, Kim MS, Whon TW, Lee MS, Bae JW. 2014. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut* **63**:727–735 DOI 10.1136/gutjnl-2012-303839.

Thong KY, McDonald TJ, Hattersley AT, Blann AD, Ramtoola S, Duncan C, Carr S, Adamson K, Nayak AU, Khurana R, Hunter SJ, Ali A, Au S, Ryder RE. 2014. The association between postprandial urinary C-peptide creatinine ratio and the treatment response to liraglutide: a multi-centre observational study. *Diabetic Medicine* **31**:403–411 DOI 10.1111/dme.12367.

Tong M, Li X, Wegener Parfrey L, Roth B, Ippoliti A, Wei B, Borneman J, McGovern DP, Frank DN, Li E, Horvath S, Knight R, Braun J. 2013. A modular organization of the human intestinal mucosal microbiota and its association with inflammatory bowel disease. *PLOS ONE* **8**:e80702 DOI 10.1371/journal.pone.0080702.

Vilsboll T, Garber AJ. 2012. Non-glycaemic effects mediated via GLP-1 receptor agonists and the potential for exploiting these for therapeutic benefit: focus on liraglutide. *Diabetes, Obesity and Metabolism* **14(Suppl 2)**:41–49 DOI 10.1111/j.1463-1326.2012.01579.x.

Wang L, Li P, Tang Z, Yan X, Feng B. 2016. Structural modulation of the gut microbiota and the relationship with body weight: compared evaluation of liraglutide and saxagliptin treatment. *Scientific Reports* **6**:33251 DOI 10.1038/srep33251.

Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, Wu Y, Schauer P, Smith JD, Allayee H, Tang WH, DiDonato JA, Lusis AJ, Hazen SL. 2011. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* **472**:57–63 DOI 10.1038/nature09922.

Weykamp C. 2013. HbA1c: a review of analytical and clinical aspects. *Annals of Laboratory Medicine* **33**:393–400 DOI 10.3343/alm.2013.33.6.393.

Wu R, Zhao D, An R, Wang Z, Li Y, Shi B, Ni Q. 2019. Linggui Zhugan formula improves glucose and lipid levels and alters gut microbiota in high-fat diet-induced diabetic mice. *Frontiers in Physiology* **10**:918 DOI 10.3389/fphys.2019.00918.

Xie Y, Bowe B, Li T, Xian H, Al-Aly Z. 2018a. Blood urea nitrogen and risk of insulin use among people with diabetes. *Diabetes and Vascular Disease Research* **15**:409–416 DOI 10.1177/1479164118785050.

Xie Y, Bowe B, Li T, Xian H, Yan Y, Al-Aly Z. 2018b. Higher blood urea nitrogen is associated with increased risk of incident diabetes mellitus. *Kidney International* **93**:741–752 DOI 10.1016/j.kint.2017.08.033.

Yachida S, Mizutani S, Shiroma H, Shiba S, Nakajima T, Sakamoto T, Watanabe H, Masuda K, Nishimoto Y, Kubo M, Hosoda F, Rokutan H, Matsumoto M, Takamaru H, Yamada M, Matsuda T, Iwasaki M, Yamaji T, Yachida T, Soga T, Kurokawa K, Toyoda A, Ogura Y, Hayashi T, Hatakeyama M, Nakagama H, Saito Y, Fukuda S, Shibata T, Yamada T. 2019. Metagenomic and metabolomic analyses reveal distinct stage-specific phenotypes of the gut microbiota in colorectal cancer. *Nature Medicine* **25**:968–976 DOI 10.1038/s41591-019-0458-7.

Yamashiro Y. 2017. Gut microbiota in health and disease. *Annals of Nutrition and Metabolism* **71**:242–246 DOI 10.1159/000481627.

Zhang Q, Xiao X, Zheng J, Li M, Yu M, Ping F, Wang T, Wang X. 2018. Featured article: structure moderation of gut microbiota in liraglutide-treated diabetic male rats. *Experimental Biology and Medicine* **243**:34–44 DOI 10.1177/1535370217743765.

Zheng J, Cheng J, Zheng S, Feng Q, Xiao X. 2018. Curcumin, a polyphenolic curcuminoid with its protective effects and molecular mechanisms in diabetes and diabetic cardiomyopathy. *Frontiers in Pharmacology* **9**:472 DOI 10.3389/fphar.2018.00472.

Zhu L, Sha L, Li K, Wang Z, Wang T, Li Y, Liu P, Dong X, Dong Y, Zhang X, Wang H. 2020. Dietary flaxseed oil rich in omega-3 suppresses severity of type 2 diabetes mellitus via anti-inflammation and modulating gut microbiota in rats. *Lipids in Health and Disease* **19**:20 DOI 10.1186/s12944-019-1167-4.