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ORIGINAL RESEARCH

The Effect of Semaglutide on Gut Microbiota in Chinese Patients with Type 2 Diabetes Poorly Controlled by Metformin

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Background: Type 2 diabetes mellitus (T2DM) is a highly prevalent metabolic disorder with increasing global incidence, linked to gut microbiota dysbiosis. This study investigated the effects of semaglutide, a long-acting GLP-1 receptor agonist, on gut microbiota composition and metabolic profiles in 15 Chinese patients with T2DM poorly controlled by metformin.

Methods: Participants received semaglutide for 12 weeks, with fecal and blood samples collected before and after treatment. 16S rRNA gene sequencing revealed significant changes in gut microbiota diversity and composition after semaglutide treatment.

Results: Alpha diversity indices increased, though not significantly, while beta diversity analysis showed structural shifts. At the phylum level, Firmicutes decreased, while Bacteroidota, Actinobacteriota and Proteobacteria increased. At the genus level, beneficial bacteria like Bifidobacterium increased, while potentially harmful genera like Klebsiella decreased. Metabolomic analysis identified 362 differentially expressed metabolites, with key pathways affected including Fc epsilon RI signaling, vascular smooth muscle contraction, and linoleic acid metabolism. Clinically, semaglutide improved glycemic control, reduced body weight, BMI and lipid. Significant correlations were observed between gut microbiota species, metabolites, and clinical indices such as BMI, HbA1c and lipid profiles.

Conclusion: Taken together, this study suggested that semaglutide's therapeutic benefits may be mediated through modulation of the gut microbiota and associated metabolic pathways, highlighting the potential for targeting the gut microbiome in diabetes management.

Keywords: type 2 diabetes mellitus, semaglutide, gut microbiota, 16S rRNA sequencing, metabolic pathways

Introduction

Type 2 diabetes mellitus (T2DM) is a highly prevalent metabolic disorder characterized by glycometabolism abnormalities, which results from abnormalities in either insulin secretion or insulin action or both. With the changes in human lifestyle, the incidence of DM has been increasing in the last decade and has become a very serious public health problem worldwide. According to a report by the International Diabetes Federation (IDF) in 2021, the number of diabetics is up to 537 million worldwide and is predicted to rise to 783 million by 2045.[1] The pathogenesis of diabetes is complicated and controversial, it is generally believed to be related to host genes, nutritional factors, physical activity, environment and gut microbiota dysbiosis.[2]

In recent years, a large number of studies have shown that altered gut microbiota composition has been associated with the occurrence, development and prognosis of diabetes.[3] The gut microbiota is a dynamic community of microorganisms made up of more than 100 trillion microbes living in the gastrointestinal tract. The gut microbiota, which is well known as the "second genome" of human body, houses a wealth of microbial cells, genomic DNA, and metabolites.[4] The microbiota of healthy adults contains a large number of normal microbiota, mainly composed of the Firmicutes, Bacteroidetes, Actinomycetes and Proteobacteria.[5] Accumulating evidences showed that there were compositional differences in the gut microbiota and their metabolic characteristics between T2DM patients and healthy individuals.[6–8]

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Further investigations have shown that many glucose-lowering drugs might alter the gut microbiome in animals and humans, including metformin,[9,10] alpha-glucosidase inhibitors (AGIs) acarbose,[11] the dipeptidyl peptidase 4 inhibitors (DPP-4i) vildagliptin[12,13] and sitagliptin,[14] the glucagon-like peptide 1 analogues (GLP-1RAs) liraglutide,[15] the sodium-glucose cotransporter 2 inhibitors (SGLT2i) dapagliflozin[16] and so on. But, with few exceptions, gliclazide and dapagliflozin treatment did not alter faecal microbiome in patients with T2DM.[17] Glucagon-like peptide 1 (GLP-1) is an incretin hormone secreted from the L cells in the gut, which has numerous physiological actions, including stimulation of insulin secretion in a glucose-dependent manner, inhibition of glucagon release, suppressing appetite, slowing gastric emptying and food intake.[18] GLP-1RAs are a well-established class of glucose-lowering agents by enhancing GLP-1 function, which effectively control glycemic levels and with a low incidence of hypoglycemia, lower body weight and blood pressure, and improve cardiovascular risk factors.[19] Previous researches reported that the alteration of gut microbiome composition is a potentially important component of the mechanisms of action of liraglutide.[20,21] Compared to liraglutide, semaglutide is a long-acting GLP-1RA with an extended half-life of approximately 1 week and permits once-weekly subcutaneous administration.[22]

However, the effect of semaglutide on gut microbiota composition in patients with T2DM poorly controlled by metformin is not fully understood. Therefore, we performed a self-controlled study in 15 patients with T2DM poorly controlled by metformin and investigate the effect of semaglutide on the composition and function of the gut microbiota through 16S rRNA gene amplicon sequencing and untargeted metabolomics analysis.

Materials and Methods

Participant

All participants were consecutively recruited from the department of Endocrinology at the Second Hospital of Hebei Medical University, a tertiary care center in Shijiazhuang, China. A total of 15 T2DM patients meeting the inclusion criteria were enrolled consecutively from January 2024 to October 2024, with a follow-up of 12 weeks for each participant. All participants were residents of Hebei Province in northern China. The regional focus Hebei Province was chosen to minimize socioeconomic and dietary heterogeneity. All procedures were performed in accordance with the Declaration of Helsinki with regard to ethical research involving human subjects and were approved by the Medical Ethics Committee of the Second Hospital of Hebei Medical University (approval ID: 2024-R0001). Written informed consents were obtained from all participants prior to enrollment.

Main inclusion criteria included the following: (1) all participants were aged 18 to 70 years; (2) all participants met the WHO criteria for overweight Asian persons (24.0–40.0 kg/m^2); (3) all participants were diagnosed with T2DM without any comorbidities, which met the 1999 World Health Organization (WHO) criteria; (4) all participants had glycated haemoglobin (HbA1c) levels ≥53.0 mmol/mol (7.0%) and ≤91.3 mmol/mol (10.5%); (5) all participants were required to have been on a stable dose of metformin (≥1.5 g/day) alone for at least 6 months before recruitment; (6) all participants maintained stable pre-study dietary habits; (7) all participants had not used any antibiotics or live biotherapeutic products within 6 months before enrollment.

Participants were excluded at the time of enrollment and at any time during the study period if any of the following exclusion criteria were met: (1) Patients with type 1 diabetes mellitus or other specific types of diabetes rather than T2DM; (2) Patients with acute complications of diabetes such as diabetic ketoacidosis, hyperosmolar hyperglycemic syndrome, or lactic acidosis within 6 months prior to enrollment; (3) Patients with serious or potentially life-threatening complications such as severe proliferative diabetic retinopathy, renal failure and diabetic foot ulcer; (4) Patients with other hypoglycemic drugs or glucocorticoids; (5) Patient with acute or chronic pancreatitis; (6) Patients with history or family history of multiple endocrine neoplasia type 2 (MEN2) or medullary thyroid cancer (MTC); (7) Patient with hyperthyroidism, Cushing's syndrome or other metabolic disease; (8) Patients with clinically significant hepatobiliary disease, glutamic aminotransferase (ALT) or glutamic oxalacetic aminotransferase (AST) >3 times the upper limit of normal; (9) Patients with moderate/severe renal impairment or end-stage renal disease, glomerular filtration rate estimate (eGFR) <60 mL/min/1.73m^2; (10) Patients with any of the following cardiovascular conditions: myocardial infarction,

cerebral infarction, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, congestive heart failure; (11) Women who are pregnant, lactating or of childbearing potential.

Study Design

The subjects with T2DM poorly controlled by metformin alone (n = 15), who met the inclusion criteria and did not meet the exclusion criteria, voluntarily participated in this study and signed the relevant written informed consents. Relevant blood and stool specimens were retained before and after 12 weeks of semaglutide treatment. Semaglutide was initiated at 0.25mg once weekly and injected subcutaneously without regard to meals. Dose escalation should occur after 4 weeks to doses of 0.5mg and the maintenance dose of 1.0mg. Patients who were unable to tolerate the gastrointestinal side effects were withdrawn from our study. Data on the age, gender, disease course, height, body weight, systolic (SBP) and diastolic blood pressure (DBP) of all participants were collected and analyzed. Body mass index (BMI) was calculated as weight/height squared (kg/m^2). The peripheral blood was collected from the subjects who fasted for 8 h, and the levels of fasting blood glucose (FBG), glycated hemoglobin (HbA1c), fasting C-peptide, triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and serum creatinine (Scr) were determined before and after semaglutide treatment. Fecal samples were collected from all participants before and after semaglutide treatment to analysis the gut microbiota. All the sample was frozen immediately at −80°C until analysis.

16S Ribosomal RNA Gene Pyrosequencing

Stool samples (n = 15) were taken just prior to commencement of semaglutide and at the end of the 12-week trial period. Stool samples were collected in sterile collection tubes, transferred into a prelabelled tube containing 8 mL of Stool DNA Stabilizer (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China), mixed by shaking and then immediately stored frozen at −80°C until being transported to the research center in chilled styrofoam containers.

Total microbial genomic DNA was extracted from 200 mg of each frozen fecal sample using the FastPure Stool DNA Isolation Kit (Magnetic bead) (MJYH, Shanghai, China) YH-feces according to the manufacturer's protocol. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (Thermo Scientific, United States). The hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3')[23] by T100 Thermal Cycler PCR thermocycler (BIO-RAD, USA). The PCR reaction mixture including 4 μL 5 × Fast Pfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL each primer (5 μM), 0.4 μL Fast Pfu polymerase, 10 ng of template DNA, and ddH_2O to a final volume of 20 μL. PCR amplification cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30s, annealing at 55°C for 30s and extension at 72°C for 45s, and single extension at 72°C for 10 min, and end at 4°C. The PCR product was extracted from 2% agarose gel and purified using the PCR Clean-Up Kit (YuHua, Shanghai, China) according to manufacturer's instructions and quantified using Qubit 4.0 (Thermo Fisher Scientific, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina PE300/PE250 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

The high-quality sequences were denoised using DADA2 plugin in the Qiime2 pipeline. DADA2 denoised sequences are usually called amplicon sequence variants (ASVs). Taxonomic assignment of ASVs was performed using the Naive Bayes consensus taxonomy classifier implemented in Qiime2 and the SILVA 16S rRNA database (v138). The metagenomic function was predicted by PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) based on ASV representative sequences. The castor was used to normalize the 16S gene copies.

Bioinformatics and Statistical Analysis

Based on the ASVs information, rarefaction curves and alpha diversity indices including observed Ace, Chao1 richness, Shannon index and Simpson index were calculated with Mothur v1.30.1. The similarity among the microbial communities in different samples was determined by principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity using Vegan v2.5–3 package. The PERMANOVA test was used to assess the percentage of variation explained by the

treatment along with its statistical significance using Vegan v2.5–3 package. The linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/LEfSe) was performed to identify the significantly abundant taxa (phylum to genera) of bacteria among the different groups (LDA score > 2, $P < 0.05$). Linear regression analysis was applied to determine the association between the clinical parameters and microbial alpha diversity indices.

Nontargeted Metabolomic Detection and Analysis

Stool samples (−80°C) were sent to the metabolomics analysis platform in Majorbio (Shanghai, China) for evaluation of nontargeted metabolomics. In brief, 50 mg stool samples were extracted with 400 μL of methanol/water (4:1, v/v) solution for 30 min at 4°C in a vortex mixer. The extract was centrifuged at 13,000 g (relative centrifugal force) for 15 min, and the supernatant was transferred. After three times of extraction, the supernatant was mixed and concentrated under nitrogen gas. Finally, samples were dissolved in 100 mL and subjected to LC-MS/MS analysis.

A pooled quality control (QC) sample was prepared by mixing equal volumes of all samples for system conditioning and QC. QC samples were prepared and tested in the same manner as analytic samples. The control represented the entire samples set and was injected after every ten samples to monitor analysis stability.

Data were analyzed with online analysis platform (Majorbio, Shanghai, China) following online instructions. Data were normalized with Pareto scaling and log-transformed before deep analysis. Principal component analysis (PCA) using data collected from positive and negative ion mode, separately, was used to reduce the complexity of the data matrix and summarized differences among groups. A supervised clustering method, partial least squares-discriminant analysis (PLS-DA), was employed to maximize variation among groups and distinguish variables useful for class separation. Variable importance in projection (VIP) values were calculated to show the contribution to distinction in the PLS-DA model. Metabolites with VIP value > 1.0 and P-value < 0.05 were the criteria used to identify variable significantly affected by semaglutide treatment.

Results

Patient Characteristics

We recruited 15 participants with T2DM poorly controlled by metformin alone to receive semaglutide for 12 weeks. The clinical characteristics of all participants before and after semaglutide treatment were presented in Table 1. The proportion of males was 33.3% (5/15), and the proportion of females was 66.7% (10/15). The average age of the participants was 55.67±8.01 years. The average duration of the disease in the participants was 6.60±4.58 years. Body weight and BMI were significantly lower in the semaglutide group than the baseline group. Fasting blood glucose and

Table 1 Therapeutic Outcomes of Type 2 Diabetic Patients Poorly Controlled by Metformin Receiving Semaglutide Treatment

	Baseline	Semaglutide
Body weight (kg)	71.62±14.52	69.03±14.41*
BMI (kg/m^2)	27.06±3.83	25.98±3.86*
HbA1c (%)	8.59±0.92	6.37±0.46*
FBG (mmol/L)	10.10±2.43	6.95±1.72*
BUN (umol/L)	5.07±1.15	5.09±1.20
Scr (mmol/L)	63.47±13.07	64.40±12.86
TC (mmol/L)	4.96±1.15	4.39±1.01*
TG (mmol/L)	1.89±0.98	1.45±0.66*
HDL (mmol/L)	1.25±0.30	1.24±0.28
LDL (mmol/L)	3.36±1.09	2.74±1.03*
SBP (mmHg)	125.0±11.40	121.3±12.16
DBP (mmHg)	84.93±7.89	82.13±8.56

Note: (Data were presented as mean ± SD, *$P < 0.05$).

HbA1c were significantly lower in the semaglutide group than the baseline group. Lipids, including TG, TC and LDL-C were significantly lower after semaglutide treatment. However, there were no notable differences observed in HDL, blood urea nitrogen and serum creatinine between the two groups. SBP and DBP were decreased, but there was no statistical difference.

16S rRNA Gene Sequencing

We used 16S rRNA gene sequencing technology to analyze the fecal microbiota composition of 15 participants before and after semaglutide treatment. The number of optimized sequences obtained from the 15 participants were 1647476 reads. The species annotation results statistics were as follows: Domain: 1; Kingdom: 1; Phylum: 12; Class: 20; Order: 46; Family: 94; Genus: 273; Species: 527; ASV: 6297.

The Changes in Gut Microbial Diversity

The Alpha diversity described the richness and diversity of species to explore the changes of gut microbiota in diabetic patient treatment with semaglutide. Ace and Chao indexes were employed to reflect richness, and Shannon and Simpson indexes were used to reflect diversity. The alpha diversity as revealed by Ace (Figure 1A), Chao (Figure 1B) and Shannon (Figure 1C) indexes were increased, and Simpson (Figure 1D) indexes was decreased after semaglutide treatment, but no statistical difference between the two groups.

The Beta diversity analysis of the gut microbiota using principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) showed that semaglutide affected the structure of the gut microbiota after semaglutide treatment. PCoA visually explores similarities and variations between the microbial compositions of two groups (Figure 1E). The results showed that the samples in the semaglutide group were separated from those in the baseline group. The total variation could be effectively explained by the first two principal axes, demonstrating an explanation rate of 14.54% (PC 1) and 9.47% (PC 2). The NMDS analysis showed the presence of different gut microbiota after semaglutide treatment with a stress value of 0.18 (Figure 1F).

The Changes in Gut Microbial Community Composition

The community bar plot can visually present the species of gut microbiota in two groups at the phylum and genus levels and the relative abundance of gut microbiota in two groups. As shown, the gut microbiota was different in two group (Figure 2).

At the phylum level, Firmicutes, Actinobacteriota, Proteobacteria and Bacteroidota were dominant bacteria in the gut microbiota (Figure 2A). There were some changes in the structural composition of the dominant bacteria after semaglutide treatment compared with the baseline group, such as a decrease in the abundance level of Firmicutes (Baseline vs Semaglutide: 73.06% vs 56.10%) and an increase in the abundance level of Actinobacteriota (14.10% vs 20.81%), Proteobacteria (10.32% vs 17.53%) and Bacteroidota (1.74% vs 5.24%) (Figure 2B). Among them, LefSe analysis revealed that the relative abundance of Firmicutes and Patescibacteria was significantly different between the baseline and semaglutide groups (Figure 2C).

At the genus level, similar changes were found, such as decreased abundance of Blautia (Baseline vs Semaglutide: 26.56% vs 6.89%) and Klebsiella (3.99% vs 3.60%), and increased abundance of Bifidobacterium (10.38% vs 17.98%), Escherichia-Shigella (3.27% vs 9.85%) and Streptococcus (0.72% vs 5.12%) after semaglutide treatment compared with the baseline group. Among them, LefSe analysis revealed that total of 24 differentially abundant bacterial taxa was found between the baseline and semaglutide groups (Figure 2D and E). The top 10 differentially abundant bacterial taxa with highest *P*-values are listed in Figure 2F.

LefSe analysis was conducted to further identify the bacterial taxa differences between two groups. As depicted in the cladogram, total of 63 differentially abundant bacterial taxa were found between the two groups, including 24 enriched and 38 depleted differential bacterial taxa after semaglutide treatment compared with the control group. At the phylum level, the relative abundance of Firmicutes and Patescibacteria were decreased after semaglutide treatment. At the class, Clostridia and Saccharimonadia were decreased, while Bacilli and Negativicutes were increased. At the Order, Actinomycetales, Corynebacteriales, Erysipelotrichales, Lachnospirales and Saccharimonadales were decreased, while Lactobacillales and

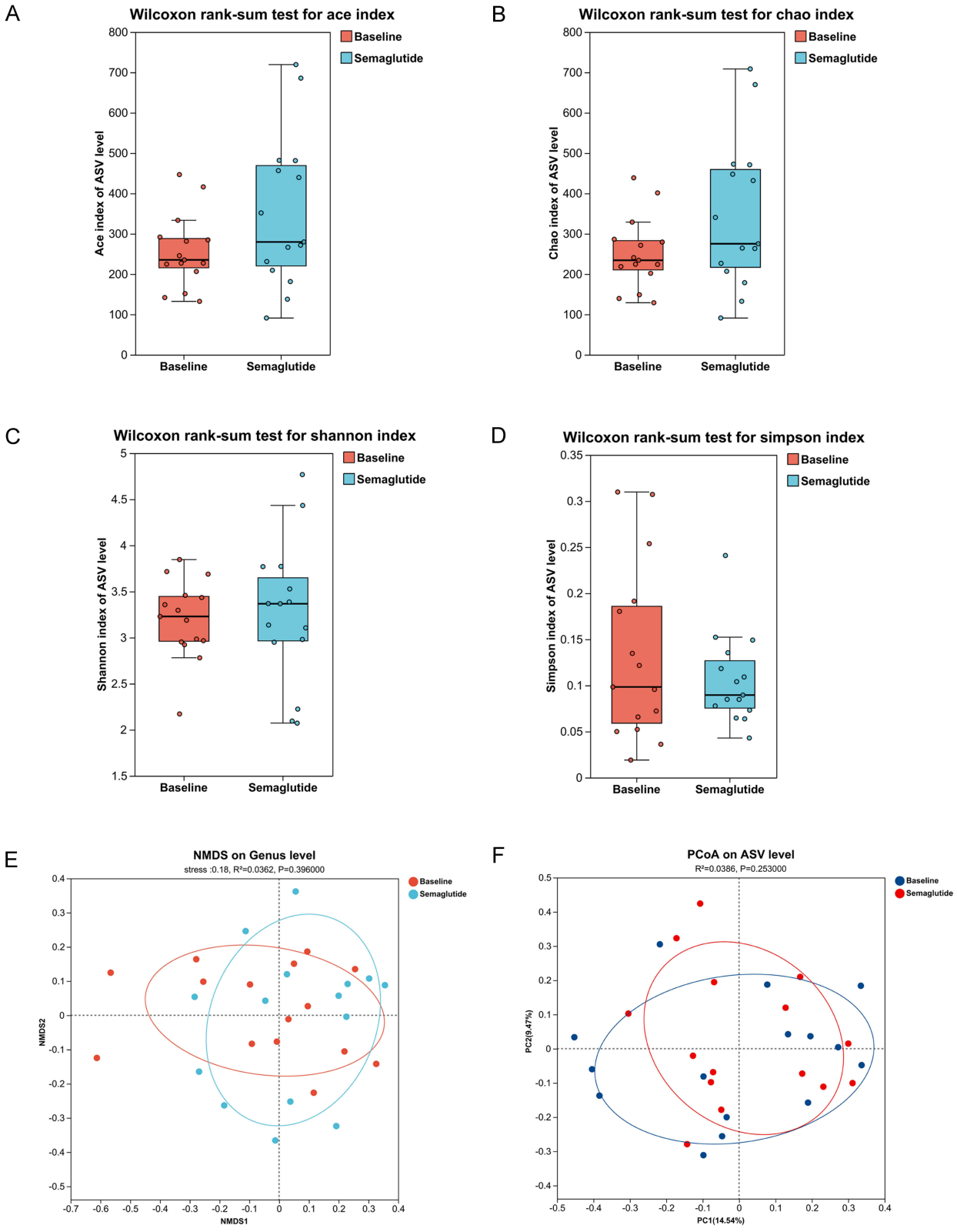

Figure 1 The gut microbial diversity of type 2 diabetic patients poorly controlled by metformin receiving semaglutide treatment. (**A**–**D**) The alpha diversity of the bacterial microbiota between the baseline and semaglutide group based on the ASV levels. (**E** and **F**) There was a difference in beta diversity between the baseline and semaglutide group by PCoA and NMDS.

Peptococcales were increased. At the family, Actinomycetaceae, Aerococcaceae, Carnobacteriaceae, Corynebacteriaceae, Erysipelatoclostridiaceae, Lachnospiraceae and Saccharimonadaceae were decreased, while Lactobacillaceae, Peptococcaceae, Streptococcaceae and UCG-010 were increased. There were 27 differentially abundant bacterial taxa at the genus, which were Actinomyces, Anaerostipes, Anaerotruncus, Blautia, Candidatus, Soleaferrea, Corynebacterium,

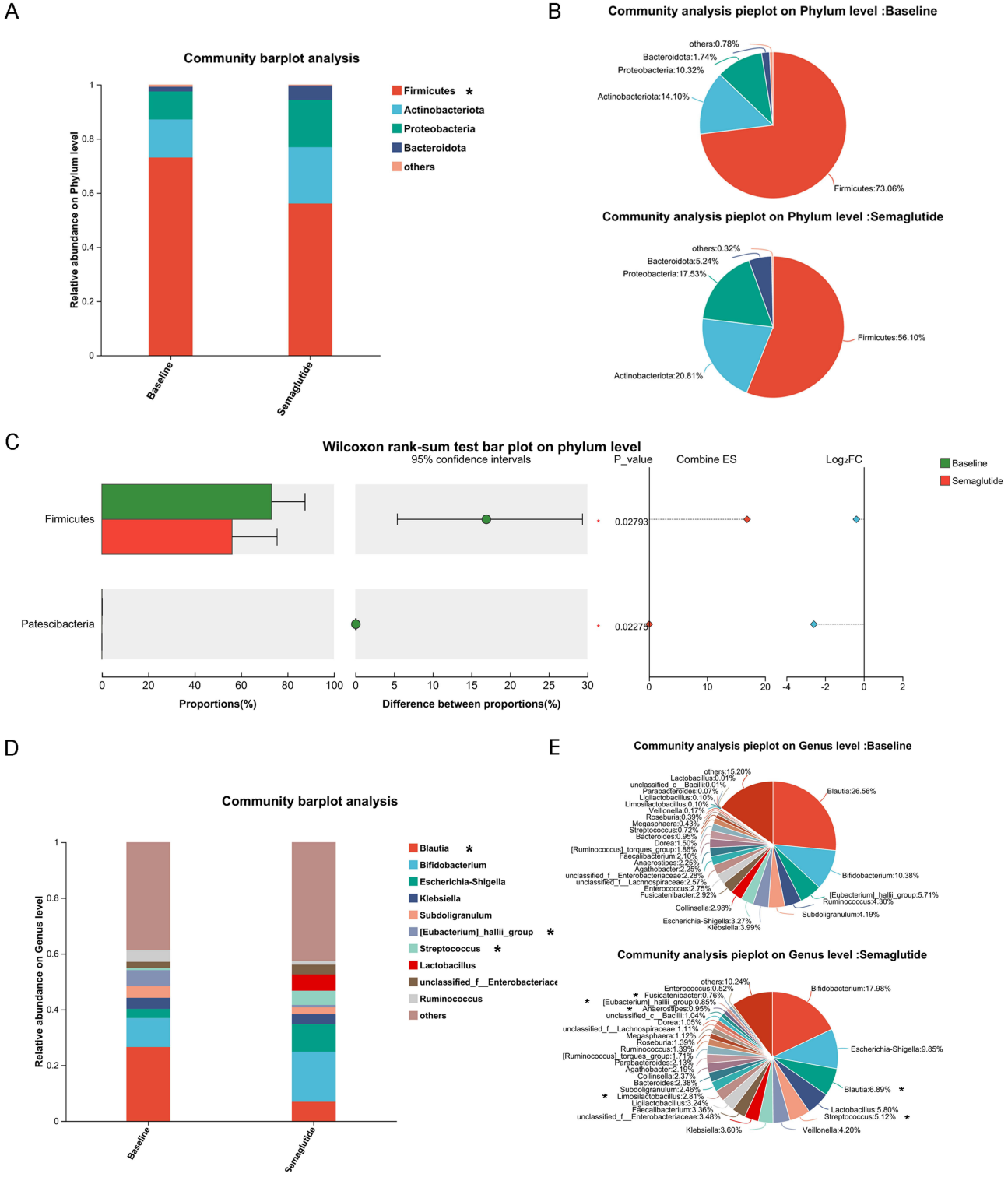

Figure 2 Continued.

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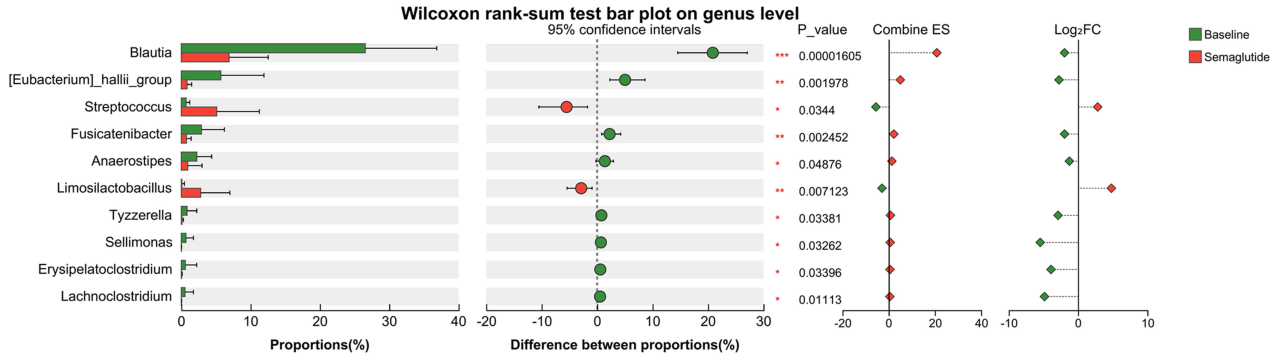

Figure 2 Microbiota composition of type 2 diabetic patients poorly controlled by metformin receiving semaglutide. (**A** and **B**) The barplot and pieplot showed the relative proportions of gut bacteria in T2DM patients poorly controlled by metformin at the phylum level. (**C**) The community bar plot showed the changes in gut microbial community composition at the phylum level between the baseline and semaglutide group. (**D** and **E**) The barplot and pieplot showed the relative proportions of gut bacteria in T2DM patients poorly controlled by metformin at the genus level. (**F**) The community bar plot showed the changes in gut microbial community composition at the genus level between the baseline and semaglutide group. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Eggerthella, Erysipelatoclostridium, F0332, Faecalitalea, Fusicatenibacter, Gordonibacter, Granulicatella, Lachnoclostridium, Lachnospiraceae, UCG-004, Lacticaseibacillus, Lactonifactor, Limosilactobacillus, Sellimonas, Streptococcus, TM7x, Tyzzerella, [Clostridium]_innocuum_group, [Eubacterium]_hallii_group, [Eubacterium]_nodatum_group (Figure 3A). The top 20 differentially abundant bacterial taxa with highest *P*-values were listed in Figure 3B.

The Relationship Between Intestinal Microbial Species and Clinical Indices

Partial Spearman's rank-based correlation test was carried out at the genus level to explore the relationship between intestinal flora and clinical variables including BMI, HbA1c, FBG, SBP, DBP, BUN, Scr, eGFR, ALT, AST, TC, TG, HDL-C, LDL-C. The data showed that BMI, HbA1c, FBG, DBP, BUN, Scr, eGFR, TC, TG, HDL-C and LDL-C were significantly correlated with the intestinal microbiota composition, while SBP, ALT and AST were not significantly correlated (Figure 4).

We found that the abundance of Limosilactobacillus, Roseburia and CAG-352 was negatively correlated with BMI. We also found that the abundance of Limosilactobacillus and CAG-352 was negatively correlated with HbA1c, while the abundance of Fusicatenibacter, Anaerostipes, Blautia, [Eubacterium]_hallii_group, Lachnoclostridium, Erysipelatoclostridium and Eggerthella was positively correlated with HbA1c. The abundance of Fusicatenibacter, Anaerostipes, Blautia, [Eubacterium]_hallii_group, [Ruminococcus]_gnavus_group, Lachnoclostridium, Erysipelatoclostridium and Eggerthella was positively correlated with FBG, while CAG-56, Christensenellaceae_R-7_group, unclassified_o_Clostridia_UCG-014, and CAG-352 was negatively correlated with FBG. The abundance of Blautia and Butyricicoccus was positively correlated with DBP. The abundance of Subdoligranulum and Streptococcus was positively correlated with BUN, while the abundance of Ruminococcus was negatively correlated with BUN. The abundance of Dorea was positively correlated with Scr, while the abundance of Subdoligranulum and Dorea was negatively correlated with eGFR. The abundance of Sellimonas was positively correlated with TC. The abundance of Fusicatenibacter, Butyricicoccus and Blautia was positively correlated with TG, while the abundance of unclassified_o__Clostridia_UCG-014 and CAG-352 was negatively correlated with TG. The abundance of Anaerostipes and Dorea was negatively correlated with HDL-C. The abundance of Sellimonas was positively correlated with TG, while the abundance of Coprococcus was negatively correlated with TG.

The Metabolic Differences After Semaglutide Treatment

A total of 1424 and 1003 metabolite components in positive and negative ion mode were determined, respectively, using LC-MS/MS based nontargeted metabolomics. The Venn diagram analyzed the composition of the baseline and semaglutide groups. As shown in Figure 5A, the number of metabolites shared between the baseline and semaglutide groups was 2747, with 309 metabolites unique to the baseline group and 145 metabolites unique to the semaglutide group.

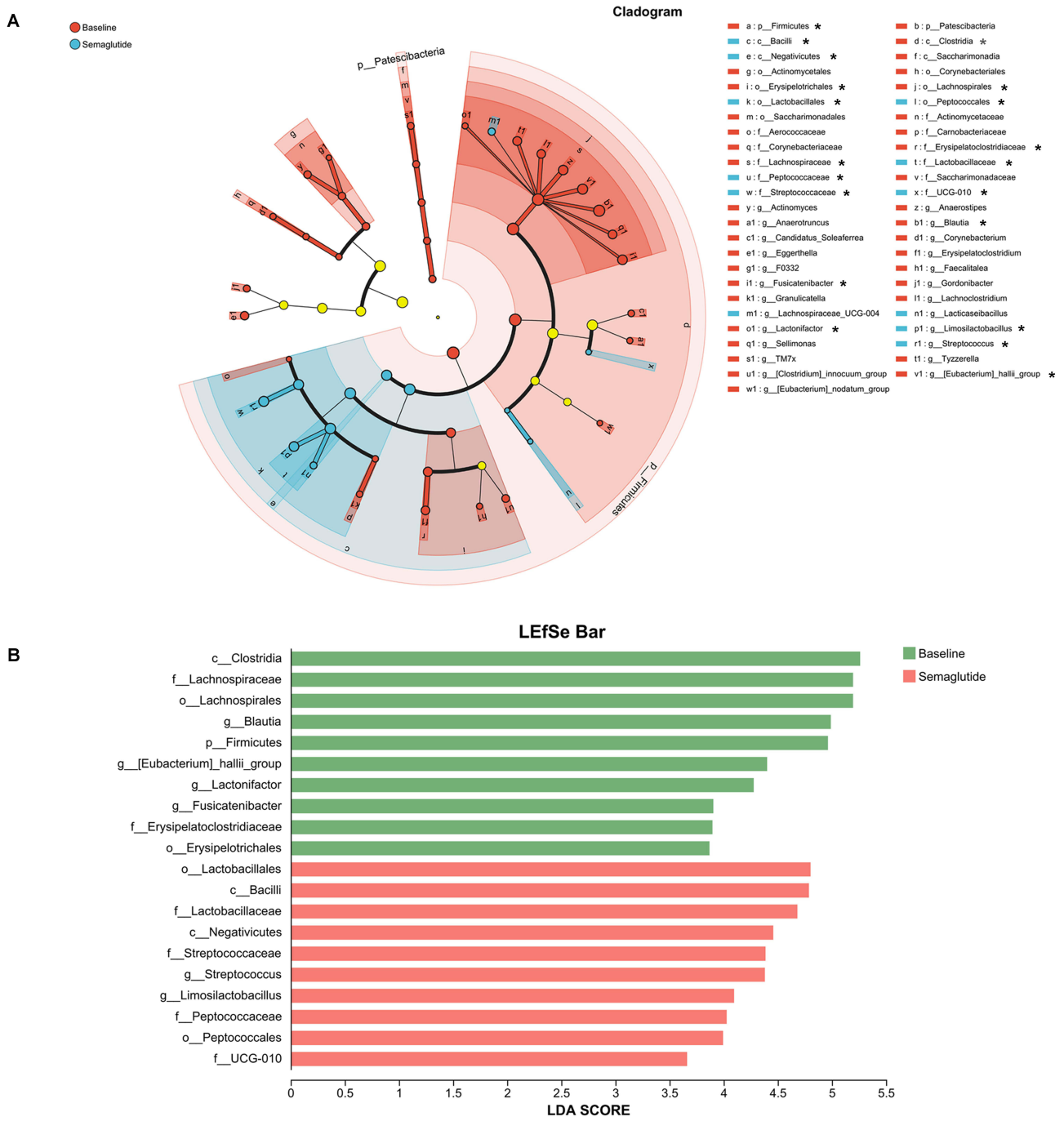

Figure 3 (**A**) Phylogenetic diagram of LEfSe analysis for the differential gut microbiota composition between the baseline and semaglutide group from the domain to species levels. (**B**) The top 20 differentially abundant bacterial taxa with highest *P*-values were listed. (*$P < 0.05$).

The PCA plot suggested varied metabolic profiles between two groups, consistent with the results of fecal microbial community structures (Figure 5B). Further, PLS-DA mode selected the most predictive and discriminative features to assist classification. The loading plot from this analysis showed clear separation in metabolites between semaglutide and baseline groups. The above results indicated that semaglutide treatment significantly altered the metabolite profiles (Figure 5C).

A total of 362 differentially expressed metabolites were identified between the semaglutide group and baseline groups, including 169 upregulated metabolites and 193 downregulated metabolites. The hierarchical clustering of differentially expressed metabolites was shown by volcano plot (Figure 5D). VIP in the PLS-DA model was calculated to examine the

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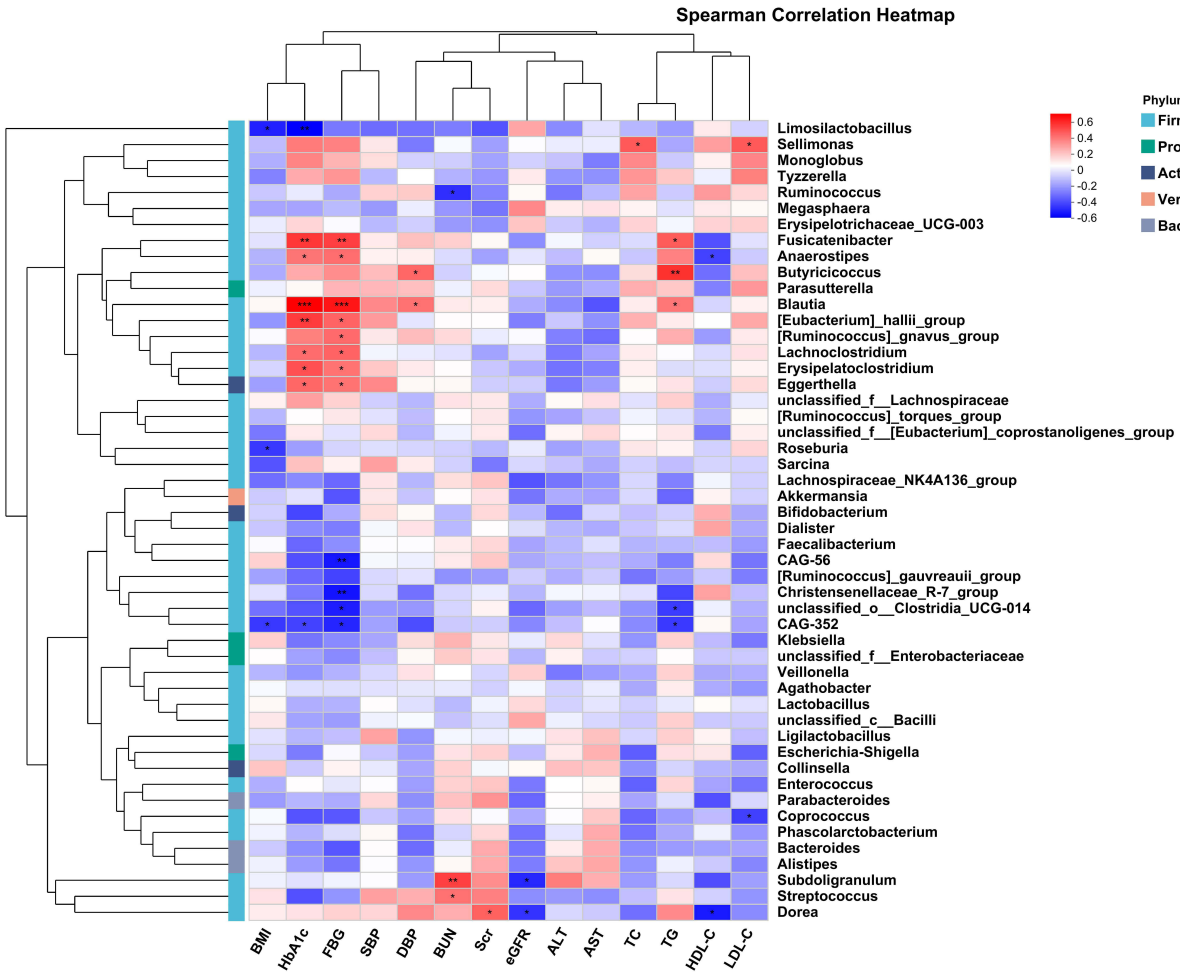

Figure 4 The heatmap showed partial Spearman correlation coefficients between the gut microbiota and the clinical indices. (*P < 0.05, **P < 0.01, ***P < 0.001).

changes in fecal metabolites in detail. Metabolites with VIP values > 1.0 and P-values < 0.05 (T-test) were considered significantly influenced by semaglutide treatment. A total of 362 differentially expressed metabolites were identified between two groups. The top 30 metabolites with highest VIP values were listed in Figure 5E. Among them, 8 identified metabolites were significantly increased, and 22 identified metabolites were decreased by semaglutide treatment.

The Metabolic Pathway Analysis of Key Differential Intestinal Metabolites

Next, KEGG enrichment analysis (Figure 6A) and differential abundance (DA) scores (Figure 6B) for the key metabolites described above were used to analyze the different metabolic pathways between the baseline and semaglutide group. We mapped all 362 differentially expressed metabolites into the KEGG database. The main enrichment pathways were Fc epsilon RI signaling pathway, Vascular smooth muscle contraction, Fc gamma R-mediated phagocytosis, Long-term depression, Parathyroid hormone synthesis, secretion and action, Inflammatory mediator regulation of TRP channels, Linoleic acid metabolism, and so on. The top 20 differentially KEGG pathway enrichment analysis and DA scores with highest P-values were listed in Figure 6A and B. The Human Metabolome Database (HMDB) database (Figure 6C) showed that lipid and lipid-like molecules (43.70%) were the main differentially expressed compounds, with organic acids and derivatives (14.66%) and phenylpropanoids and polyketides (9.68%) being represented.

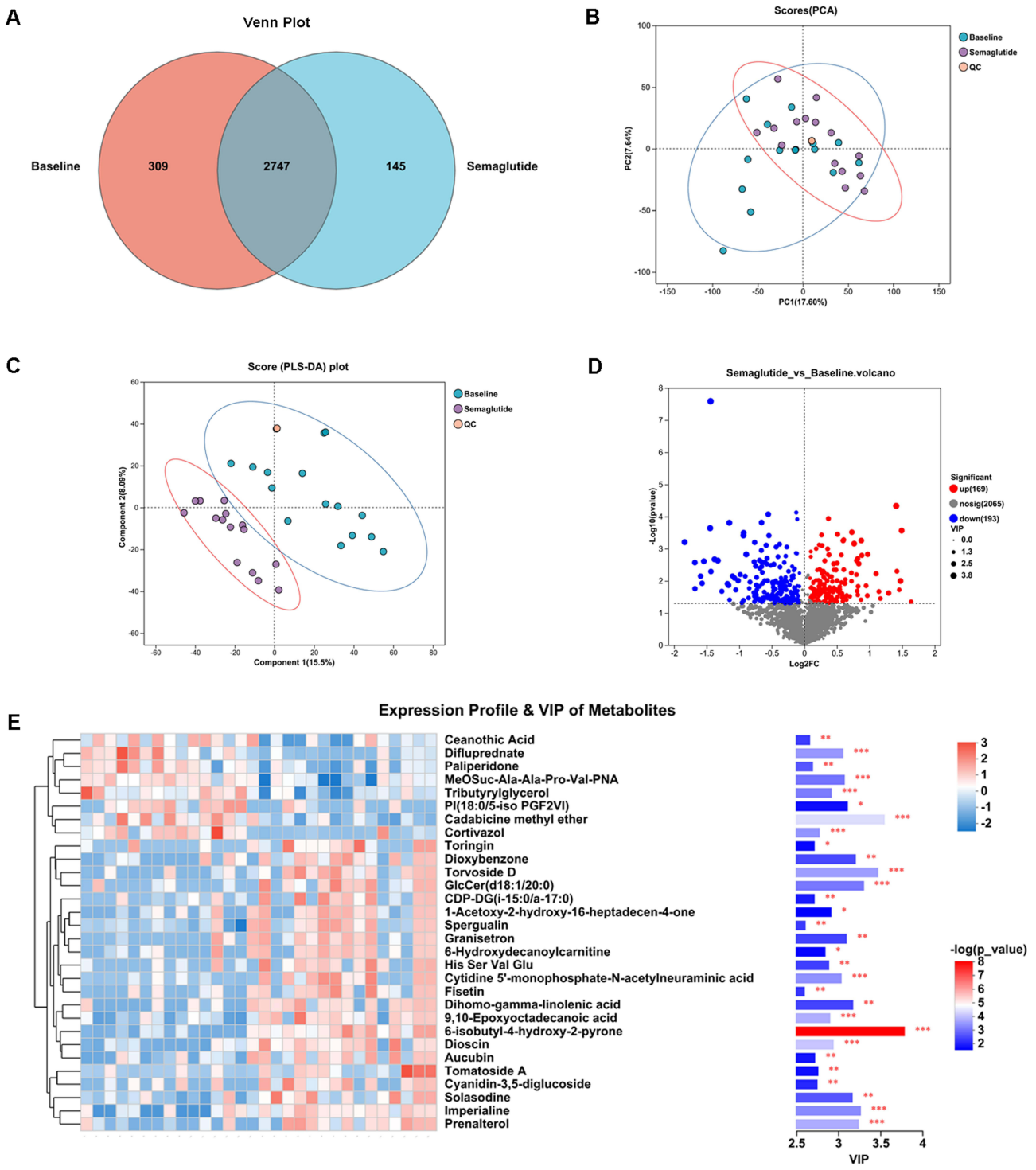

Figure 5 (**A**) Venn diagram of differential metabolites between the baseline and semaglutide group. Different colors represent different subgroups, with overlapping numbers representing the number of metabolites common to multiple subgroups and nonoverlapping numbers representing the number of metabolites specific to the corresponding subgroup. (**B** and **C**) The PCA plot and PLS-DA plot showed the varied metabolic profiles between the baseline and semaglutide group. (**D**) The volcano plot showed the hierarchical clustering of differentially expressed metabolites between semaglutide and baseline groups. (**E**) The top 30 differentially metabolites with highest VIP values were listed. (*P < 0.05, **P < 0.01, ***P < 0.001).

The Relationship Between Metabolites and Clinical Indices

Further, Partial Spearman's rank-based correlation test was carried out to explore the relationship between metabolites and clinical variables including BMI, HbA1c, FBG, SBP, DBP, BUN, Scr, eGFR, ALT, AST, TC, TG, HDL-C, LDL-C.

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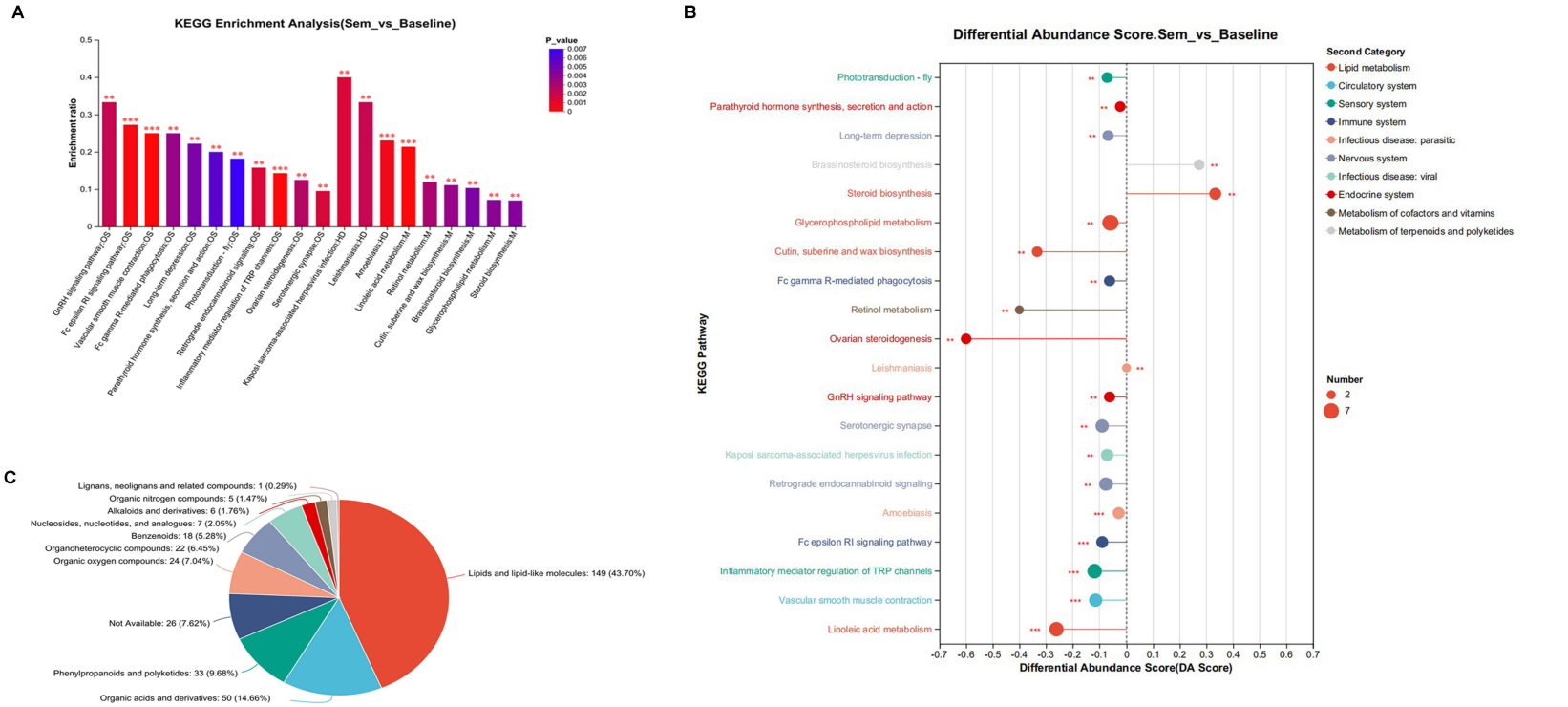

Figure 6 (**A**) KEGG differential metabolite enrichment analysis between the baseline and semaglutide group. The top 20 differentially KEGG pathway enrichment analysis with highest P-values were listed. (**B**) The DA scores of differential metabolites between the baseline and semaglutide group. The top 20 differentially DA scores with highest P-values were listed. (**C**) The Human Metabolome Database (HMDB) superclass compound classification of differential metabolites between the baseline and semaglutide group. (***P* < 0.01, ****P* < 0.001).

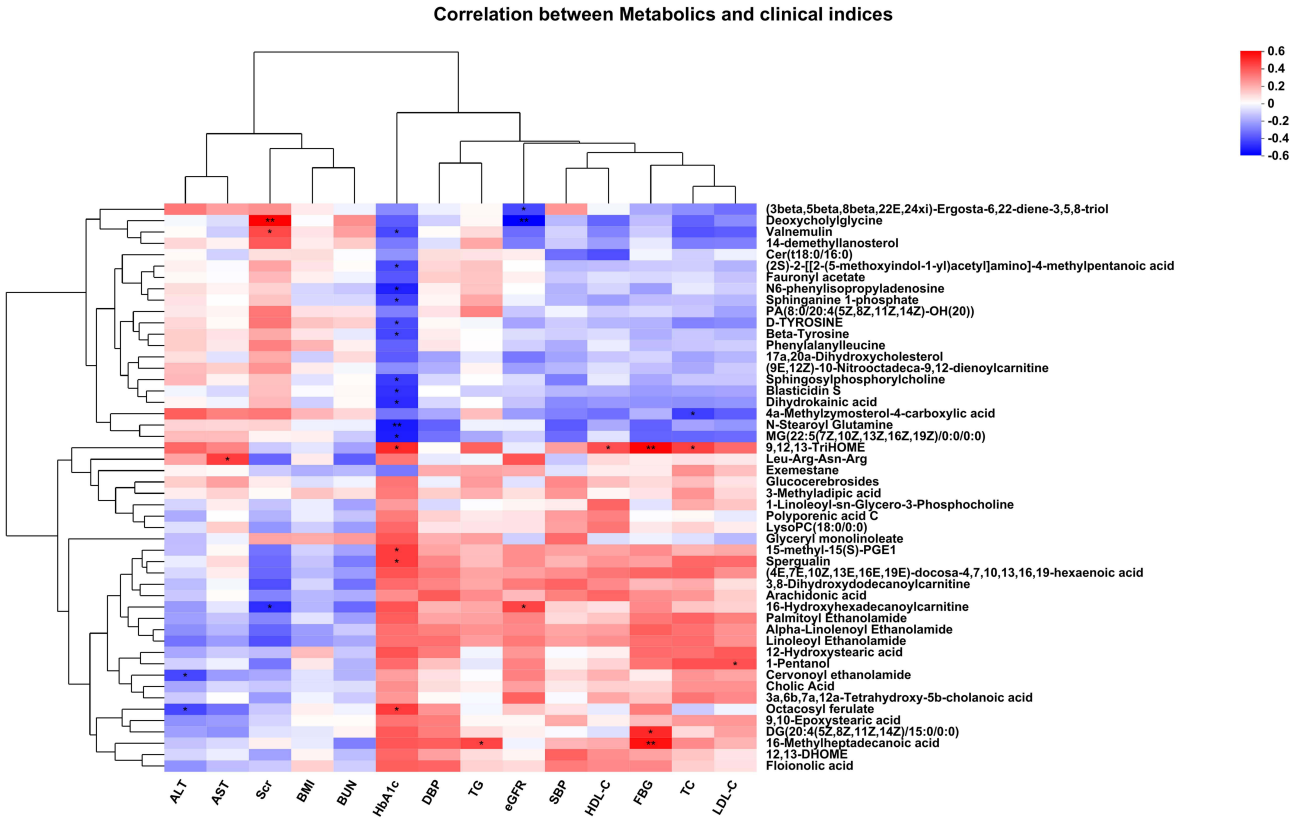

Figure 7 The heatmap showed partial Spearman correlation coefficients between the differential metabolite and the clinical indices. (*P < 0.05, **P < 0.01).

The data showed that HbA1c, FBG, BUN, Scr, eGFR, TC, TG, HDL-C and LDL-C were significantly correlated with the metabolites, while DBP, SBP, BMI and BUN were not significantly correlated (Figure 7).

We found that the expression of Cervonoyl ethanolamide Octacosyl ferulate was negatively correlated with ALT, while the expression of Leu-Arg-Asn-Arg was positively correlated with AST. We also found that the expression of Deoxycholylglycine and Valnemulin was positively correlated with Scr, while the expression of 16-Hydroxyhexadecanoylcarnitine was negatively correlated with Scr. The expression of 9,12,13-TriHOME, 15-methyl-15 (S)-PGE1, Spergualin and Octacosyl ferulate was positively correlated with HbA1c, while the expression of Valnemulin, (2S)-2-[[2-(5-methoxyindol-1-yl)acetyl]amino]-4-methylpentanoic acid, N6-phenylisopropyladenosine, Sphinganine 1-phosphate, D-TYROSINE, Sphingosylphosphorylcholine, Blasticidin S, Dihydrokainic acid, N-Stearoyl Glutamine, MG(22:5(7Z,10Z,13Z,16Z,19Z)/0:0/0:0) and Beta-Tyrosine was negatively correlated with HbA1c. The expression of 16-Methylheptadecanoi acid was positively correlated with TG. The expression of (3beta,5beta,8beta,22E,24xi)-Ergosta-6,22-diene-3,5,8-triol and Deoxycholylglycine was negatively correlated with eGFR, while the expression of 16-Hydroxyhexadecanoylcarnitine was positively correlated with eGFR. The expression of 9,12,13-TriHOME was positively correlated with HDL-C. The expression of 9,12,13-TriHOME, DG (20:4(5Z,8Z,11Z,14Z)/15:0/0:0) and 16-Methylheptadecanoic acid was positively correlated with FBG. The expression of 4a-Methylzymosterol-4-carboxylic acid was negatively correlated with TC, while the expression of 9,12,13-TriHOME was positively correlated with TC. The expression of 1-Pentanol was positively correlated with LDL-C.

Discussion

This study investigated the effect of semaglutide on gut microbiota composition and metabolic profiles in Chinese patients with T2DM poorly controlled by metformin. The findings suggested that semaglutide treatment not only improved glycemic control but also induced significant changes in gut microbial diversity and community structure, as well as alterations in metabolic pathways.

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In our current study, semaglutide treatment resulted in significant reductions in body weight, BMI, FBG, HbA1c, and the lipid levels (TG, TC, LDL-C), consistent with its known glucoregulatory and metabolic benefits.[24] Notably, SBP and DBP showed a trend toward reduction, though without statistical significance. These improvements are likely mediated by semaglutide's actions on glucose-dependent insulin secretion, appetite suppression and weight loss.

The 16S rRNA sequencing analysis revealed that semaglutide treatment altered the gut microbiota composition at both the phylum and genus levels. Alpha diversity indices were increased after semaglutide treatment, indicating enhanced microbial richness and diversity, although the changes were not statistically significant. Maybe the high intra-group variability likely contributed to the lack of statistical significance. Beta diversity analysis confirmed structural changes in the gut microbiota, further supporting the impact of semaglutide. Previous study also showed that alpha diversity and beta diversity of T2DM patients treated with metformin combined with liraglutide significantly differed from that of T2DM patients treated with metformin alone.[25]

In our current study, semaglutide treatment could decrease the abundance of Firmicutes and increase the abundance of Bacteroidota, Actinobacteriota and Proteobacteria. Previous studies have shown that the increase of Firmicutes/Bacteroidetes ratio could decrease the production of short-chain fatty acids (SCFA) and increase energy intake.[26,27] Firmicutes are linked to efficient energy harvest via producing SCFA, which contributes to fat storage and weight gain.[28] Notably, many studies on GLP-1RAs found a reduction in Firmicutes,[29–31] indicating that GLP-1 has a universal inhibitory effect on this obesity-associated taxon. Bacteroidota species produce propionate, a SCFA, that suppresses hepatic lipogenesis and enhances satiety signals, synergizing with semaglutide effects to reduce energy intake and promote fat oxidation.[32] The shifts of Firmicutes and Bacteroidota aligned with semaglutide-induced weight loss by limiting excessive caloric absorption. Semaglutide treatment also could increase the abundance of Actinobacteriota. Actinobacteriota can enhance the ability of carbohydrate degradation, increase the production of SCFAs and reduce fat accumulation.[33] Actinobacteriota-derived acetate also may enhance insulin sensitivity via G protein-coupled receptor (GPCR) signaling in adipose tissue and the liver.[34] Acetate also suppresses appetite through hypothalamic pathways, complementing GLP-1RA-induced satiety.[35] Compared with previous study on liraglutide,[25] liraglutide did not alter the abundance of Actinobacteriota. These differences suggested that semaglutide treatment may offer additional metabolic advantages through unique microbiota-metabolism axis modulation, potentially explaining their enhanced clinical performance. Proteobacteria encompass many opportunistic pathogens, which thrive in inflamed or dysbiotic environments.[36] Their expansion may indicate subclinical gut inflammation or disrupted microbial balance, potentially counteracting the metabolic benefits of GLP-1RAs. Elevated Proteobacteria are correlated with increased intestinal permeability and endotoxemia,[37] which drive chronic low-grade inflammation, insulin resistance, and cardiovascular risk. While GLP-1RAs generally improve metabolic parameters, a Proteobacteria-rich microbiome might undermine these effects by promoting inflammation or altering bile acid metabolism. Maybe this could explain interindividual variability in treatment responses.

At the genus level, the abundance of Bifidobacterium was significantly increased. Bifidobacterium, as a well-known beneficial genus, could produce lactate and acetate, support gut barrier function and modulate immune responses. Increased Bifidobacterium abundance could enhance SCFA production and reduce systemic inflammation, which could improve glucose tolerance and insulin sensitivity.[38] Bifidobacterium also can lower serum cholesterol levels by metabolizing bile acids and reducing lipid absorption in the gut.[39] The abundance of Klebsiella was obviously decreased. Klebsiella is often associated with opportunistic infections and can produce harmful metabolites, such as ethanol and LPS, which may disrupt gut homeostasis. Decreased Klebsiella levels could improve glucose metabolism by reducing inflammation and insulin resistance. Ethanol production by Klebsiella may also interfere with liver function and glucose regulation. Klebsiella overgrowth may contribute to dyslipidemia by increasing systemic inflammation and altering bile acid metabolism.[40] The reduction of Klebsiella levels following semaglutide treatment may play a significant role in improving glucose and lipid metabolism. These results confirmed that semaglutide treatment modulated gut microbiota, reduced the abundance of harmful intestinal bacteria and increased the abundance of beneficial intestinal bacteria.

Metabolomic analysis identified 362 differentially expressed metabolites between the baseline and semaglutide groups, with key pathways affected including Fc epsilon RI signaling, vascular smooth muscle contraction, and linoleic

acid metabolism. The enrichment of lipid and lipid-like molecules, organic acids, and phenylpropanoids suggests that semaglutide may modulate metabolic processes related to inflammation, lipid metabolism, and energy homeostasis.

Significant correlations were found between gut microbiota species and clinical indices, particularly BMI, HbA1c, FBG, and lipid profiles. Similarly, metabolites were correlated with key clinical variables, highlighting the interconnectedness of gut microbiota, metabolism, and clinical outcomes. These findings underscore the potential role of the gut microbiome in mediating the therapeutic effects of semaglutide.

The observed shifts in gut microbiota composition and metabolic pathways following semaglutide treatment offered promising clinical implications for T2DM treatment. The increase in beneficial bacteria (eg, Bifidobacterium, Bacteroidota) and reduction in potentially harmful genera (eg, Klebsiella) suggested that semaglutide might exert part of its metabolic benefits through microbiome modulation. These changes could enhance gut barrier function, reduce systemic inflammation, and improve insulin sensitivity, complementing the drug's direct glucoregulatory effects. Clinically, monitoring gut microbiota composition might help predict treatment response, particularly in patients with poor glycemic control despite metformin therapy. Additionally, combining semaglutide with probiotics or dietary interventions targeting microbial balance (eg, high-fiber diets) could further optimize metabolic outcomes.

Conclusion

In conclusion, semaglutide treatment in T2DM patients poorly controlled by metformin led to significant clinical improvements, accompanied by notable changes in gut microbiota composition and metabolic profiles. The observed shifts in microbial diversity and abundance, along with alterations in metabolic pathways, suggest that semaglutide may exert its benefits through modulation of the gut microbiome and associated metabolic processes. These findings provide novel insights into the mechanisms underlying semaglutide's therapeutic effects and highlight the potential for targeting the gut microbiota in diabetes management.

However, the study has several limitations that warrant consideration. While we instructed patients to maintain their habitual diets during the study, we acknowledge that semaglutide's appetite-modulating effects may have unconsciously altered their dietary patterns, potentially confounding our microbiota observations. The small sample size may limit the generalizability of the findings, and the lack of a control group makes it difficult to distinguish semaglutide-specific effects from natural microbiome fluctuations over time. Furthermore, the absence of long-term follow-up precludes conclusions about the durability of these microbial and metabolic changes. Future studies should incorporate larger, randomized controlled trials with longitudinal follow-up to validate these findings. Despite these limitations, our study provided preliminary evidence that gut microbiota modulation might be a novel mechanism underlying semaglutide's therapeutic effects, opening new avenues for personalized T2DM management.

Disclosure

The authors report no conflicts of interest in this work.

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