



# Changes in the Microbiota and their Roles in Patients with Type 2 Diabetes Mellitus

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Received: 10 March 2022 / Accepted: 10 February 2023 / Published online: 9 March 2023  
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## Abstract

An association between type 2 diabetes mellitus (T2DM) and gut microbiota is well established, but the results of related studies are inconsistent. The purpose of this investigation is to elucidate the characteristics of the gut microbiota in T2DM and non-diabetic subjects. Forty-five subjects were recruited for this study, including 29 T2DM patients and 16 non-diabetic subjects. Biochemical parameters, including body mass index (BMI), fasting plasma glucose (FPG), serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), and hemoglobin A1c (HbA1c), were analyzed and correlated with the gut microbiota. Bacterial community composition and diversity were detected in fecal samples using direct smear, sequencing, and real-time polymerase chain reaction (PCR). In this study, it was observed that indicators such as BMI, FPG, HbA1c, TC, and TG in T2DM patients were on the rise, concurrent with dysbiosis of the microbiota. We observed an increase in *Enterococci* and a decrease in *Bacteroides*, *Bifidobacteria*, and *Lactobacilli* in patients with T2DM. Meanwhile, total short-chain fatty acids (SCFAs) and D-lactate concentrations were decreased in the T2DM group. In addition, FPG was positively correlated with *Enterococcus* and negatively correlated with *Bifidobacteria*, *Bacteroides*, and *Lactobacilli*. This study reveals that microbiota dysbiosis is associated with disease severity in patients with T2DM. The limitation of this study is that only common bacteria were noted in this study, and more in-depth related studies are urgently needed.

## Introduction

T2DM is a chronic metabolic disease characterized by insulin resistance (IR) and relatively inadequate insulin secretion [1]. The global prevalence of diabetes is estimated to be 9.3% (463 million people) by 2019, rising to 10.2% (578 million people) by 2030 and 10.9% (700 million people) by 2045. The common complications of diabetes include macroangiopathy, microvascular lesions, diabetic nephropathy,

diabetic retinopathy, and peripheral neuropathy [2]. Genetic factors, high caloric intake, and physical inactivity are recognized as major risk factors for T2DM [3]. Currently, the association between gut microbiota and T2DM has been subjected to intense discussion.

The human gut microbiota consists of several types of microorganisms, including bacteria, archaea, eukaryotes, viruses, and parasites. More than  $1 \times 10^{14}$  microorganisms have been identified in the human gastrointestinal tract, including seven major branches, namely, the phyla *Firmicutes*, *Bacteroidetes*, *Actinomycetes*, *Chondrobacteria*, *Proteobacteria*, *Mollusculaceae*, and *Cyanobacteria*, with *Firmicutes* and *Bacteroidetes* being the most dominant [4]. Under normal conditions, the microbiota structure remains in dynamic balance and plays a key role in energy metabolism and nutrition regulation [5]. A study showed that germ-free (GF) mice had a lower incidence of obesity than conventionally raised donor high-fat diet (HFD)-induced mice, yet gained weight after transplantation of fecal microbiota from obese mice [6]. The microbiota influences T2DM by modulating inflammation and interacting with dietary components. It is well known in the literature that fermentation

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products of the microbiota can influence intestinal permeability, glucose and lipid metabolism, insulin sensitivity, and overall energy homeostasis [7]. However, changes in diet, drug exposure, or infection may cause dysbiosis, which can lead to metabolic diseases such as obesity and diabetes [8]. A cross-sectional study of patients versus controls reported that an elevated abundance of *Lactobacilli* aggravated IR and T2DM [9]. On the other hand, *Lactobacillus fermentum*, *Plantarum* and *casei*, *Roseburia intestinalis*, *Akkermansia muciniphila*, and *Bacteroides fragilis* have all been shown to improve glucose metabolism and insulin sensitivity and suppress pro-inflammatory cytokines [10]. Metformin, used to treat diabetes, has been shown to interact with the gut microbiota by modulating glucose metabolism, increasing SCFAs, and enhancing intestinal permeability to lipopolysaccharides (LPS) [11]. In clinical and animal studies, probiotics have been reported to prevent or delay the onset of diabetes by repairing the intestinal barrier, suppressing inflammatory responses, reducing oxidative stress, restoring energy metabolism, and producing beneficial microbial metabolites including SCFAs and bile acids [12].

Acetate, propionate, and butyrate are SCFAs produced by the fermentation of indigestible carbohydrates, which have positive effects on energy metabolism, intestinal homeostasis, and control of the immune response. SCFAs increase lipocalin and insulin production by promoting the release of glucagon-like peptide-1 (GLP-1) and GLP-2 hormones, thereby improving insulin sensitivity and islet cell proliferation [13]. Several species of *Bifidobacterium* and *Lactobacillus* showed beneficial effects on T2DM by reducing plasma lipids, and pro-inflammatory genes and increasing the production of SCFAs [14]. Besides, butyrate supplementation has been reported to improve glycemic control and insulin resistance in C57BL/6 J mice [12].

Previous studies have focused on the relationship between gut microbiota and diabetes in animal models rather than in humans. Animal models offer useful insights that cannot be carried out in humans [15]. Preclinical studies have shown that some bacteria have a direct effect on certain metabolic and clinical parameters of diabetes, but clinical trials are few and the results are not promising. The current literature on the microbiota of T2DM is inconsistent and there are concerns about the variability of results. Currently, the pressing issue is to identify the microbiota and host factors that may indeed contribute to this apparent lack of data reproducibility. Thus, this project was conceived to test and verify whether similar changes would be observed in patients with T2DM. We used direct smear microscopy, high-throughput sequencing, quantitative real-time PCR, SCFAs, and D-lactate assays to study and compare the structure of the fecal bacterial microbiota in T2DM patients and healthy persons based on animal pre-experiments and some related studies. We aimed to open a new pathway for the

diagnosis, treatment, and prognosis of T2DM through the above methods.

## Materials and Methods

### Subjects

Twenty-nine patients who were diagnosed with T2DM were enrolled in this study. The inclusion criteria were as follows: (1) They had not taken antibiotics, micro-ecological preparations, and other drugs that could influence microbiota within 1 month before collecting stool specimens. (2) There was no medical history of diarrhea, intestinal infection, or other gastrointestinal diseases in the past 4 weeks. (3) There was no serious damage to organs such as the heart, liver, and kidney or any tumor diseases. (4) There were no other endocrine diseases such as thyroid diseases. (5) Fecal examinations were normal (no infection). (6) There was no pharmacological intervention for T2DM patients. In addition, incomplete clinical data were excluded. The control group of 16 subjects was drawn from a pool of healthy volunteers whose routine physical examinations were normal. Neither the experimental group nor the control group received drug treatment and had no history of gastrointestinal diseases such as diarrhea in the past 4 weeks and no history of chronic gastrointestinal diseases.

### Anthropometry and Collection of Fecal Specimens

We calculated BMI based on the height and weight of each subject. Fresh fecal specimens from the control and T2DM groups were placed in sterile containers and then moved within 0.5 h to a  $-80^{\circ}\text{C}$  freezer.

### Detection of Biochemical Indices

Two tubes of fasting venous blood samples were collected and centrifuged to collect serum samples, and then high-performance liquid chromatography (HPLC) was adopted to detect HbA1c. The Hitachi 7180 automatic biochemical instrument was used to detect FPG, TC, TG, HDL, and other indices within 2 h. Finally, the data were recorded.

### Microscopic Examination of Intestinal Bacteria

The fecal specimens stored in the freezer at  $-80^{\circ}\text{C}$  were slowly thawed, 0.1 g of feces was taken for a direct routine smear, and then the samples were dried, fixed, and subjected to Gram staining. Finally, 10 fields of view were selected to observe bacteria under an oil microscope. The smears were classified into three degrees of dysbiosis according to the degree of alteration in the number

and proportion of microbiota, and the "Examination chart of microbiota stool smear" can be used as a reference to determine the degree of dysbiosis of the intestinal microbiota.

### DNA Extraction, Amplification, Sequencing and Sequence Analysis

Sequencing was performed using a  $2 \times 300$  paired-end configuration, and the data were analyzed using MiSeq Control software (version 2.5.0.5; Illumina, Inc.). To process the raw data, all the forward and reverse reads were assembled in pairs, the sequences containing N in the results were filtered, and sequences  $> 200$  bp in length were retained. Subsequently, the spliced and filtered sequences were compared with the Basic Local Alignment Search Tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) match lengths (against reference sequences in the database) to remove the chimera sequence ( $< 90\%$  match). In the operational taxonomic unit (OTU) analysis, the 16S reads were clustered using VSEARCH software (version 1.9.6; <https://github.com/torognes/vsearch>) with a pairwise identity cutoff of 97%, and the 16S rRNA reference database was Silva 132 (<https://www.arb-silva.de/search/>). Representative sequences of each OTU were analyzed using the Ribosome Database Program classifier (version 2.2, <http://rdp.cme.msu.edu/classifier/classifier.jsp;jsessionid=D5D6C78C6C197C015E237D0FD7A85246.10.0.0.9>). The Bayesian algorithm and the composition of each sample at different species classification levels were calculated. According to the OTU analysis results, the random sampling method was used to calculate the  $\alpha$  diversity index of Shannon and Chao1, and the dilution curve was obtained. Unweighted UniFrac analysis was applied to compare whether there were significant differences in microbial communities among samples. The Bray-Curtis distance matrix among samples was used for PCoA (principal coordinate analysis) to indicate  $\beta$  diversity, and the P value was calculated using a nonparametric MANOVA. To compare the hierarchical relationships among groups, the unweighted pair group method for the arithmetic mean (UPGMA) clustering tree was constructed by the nonweighted mean method in hierarchical clustering. Anonym analysis was confirmed according to Bray-Curtis. Linear discriminant effect size analysis was performed by linear discriminant analysis (LDA; [huttenhower.sph.harvard.edu/galaxy](http://huttenhower.sph.harvard.edu/galaxy)) to calculate the impact of species richness on the different effects and to identify the species with a

significant difference. Based on the  $\beta$  diversity distance matrix and environmental factor data, redundancy analysis was performed.

### Quantitative Transcript Analysis

Total RNA was extracted from fecal samples using TRIzol reagent (Invitrogen, USA) and cDNA was synthesized using Revert Aid First Strand cDNA Synthesis kit (TaKaRa, Japan). cDNA was amplified in triplicate using gene-specific primers (Table S1), using Power Up SYBR Green (Thermo Scientific, USA), and using the 7500 Fast Real-Time PCR machine (Applied Biosystems), and mRNA levels were measured in triplicate. RT-qPCR was performed under the following conditions: Enzyme activation at 95 °C for 3 min, followed by denaturation at 95 °C for 20 s, primer annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. Samples were cooled to 65 °C, then heated to 95 °C in steps of 0.05 °C, and melting curves were determined. All RT-qPCR data were normalized to  $\beta$ -actin expression.

### Determination of the SCFAs in Feces

The concentration of SCFAs was detected by gas chromatography. The stool sample (300 mg) was combined with 0.4 mL of ether, 0.1 mL of phosphoric acid, and 0.1 mL of isotropic acid. The supernatant was brought to the machine for testing after being centrifuged at 4 °C for 10 min at 12,000 rpm. The pure standards of acetic acid, propionic acid, n-butyric acid, and 2-methyl butyric acid were measured and formed into mixed standard concentration gradients at concentrations of 0.05  $\mu\text{g/mL}$ , 0.1  $\mu\text{g/mL}$ , 0.5  $\mu\text{g/mL}$ , 1  $\mu\text{g/mL}$ , 5  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , 25  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$ , and 250  $\mu\text{g/mL}$ , with a split injection volume of 1  $\mu\text{L}$ , and a splitting ratio of 10:1, a flame ionization detector, and a capillary column (Agilent HP-INNOWAX, 30 m, 0.25 mm, 0.25  $\mu\text{m}$ ) were utilized. The injection port was 250 °C; ion source temperature was 230 °C; the transmission line temperature was 250 °C, quadrupole temperature was 150 °C. The programmed temperature was increased from 90 °C to 120 °C at 10 °C/min, then to 150 °C at 5 °C/min, and finally to 250 °C at 25 °C/min for 2 min. The carrier gas was helium with a flow rate of 1.0 mL/min.

## Detection of the D-Lactic Acid in Feces

The concentration of D-Lactic acid was measured by the ultraviolet assay. A total of 0.15 g of the sample was precisely weighed, and water was added to make it up to 1.0 ml. The mixture was mixed well to make a suspension and centrifuged at 15,000 rpm for 3 min, and the supernatant was collected to detect D-lactic acid. The specific steps were performed according to the kit instructions. The D-lactic acid content was determined by measuring the change in the absorbance value of NADH at 340 nm.

## Multivariate Statistical Analysis

Statistical analysis was performed using SPSS software (version 19). The quantitative data was expressed as the mean  $\pm$  standard error (mean  $\pm$  SE). A T-test was used for comparisons between the two groups, and Pearson correlation analysis was used to analyze the correlation between FPG and HbA1c and *Enterococcus*, *Bacteroides*,

*Bifidobacillus*, and *Lactobacillus*. A difference of  $P < 0.05$  was considered statistically significant.

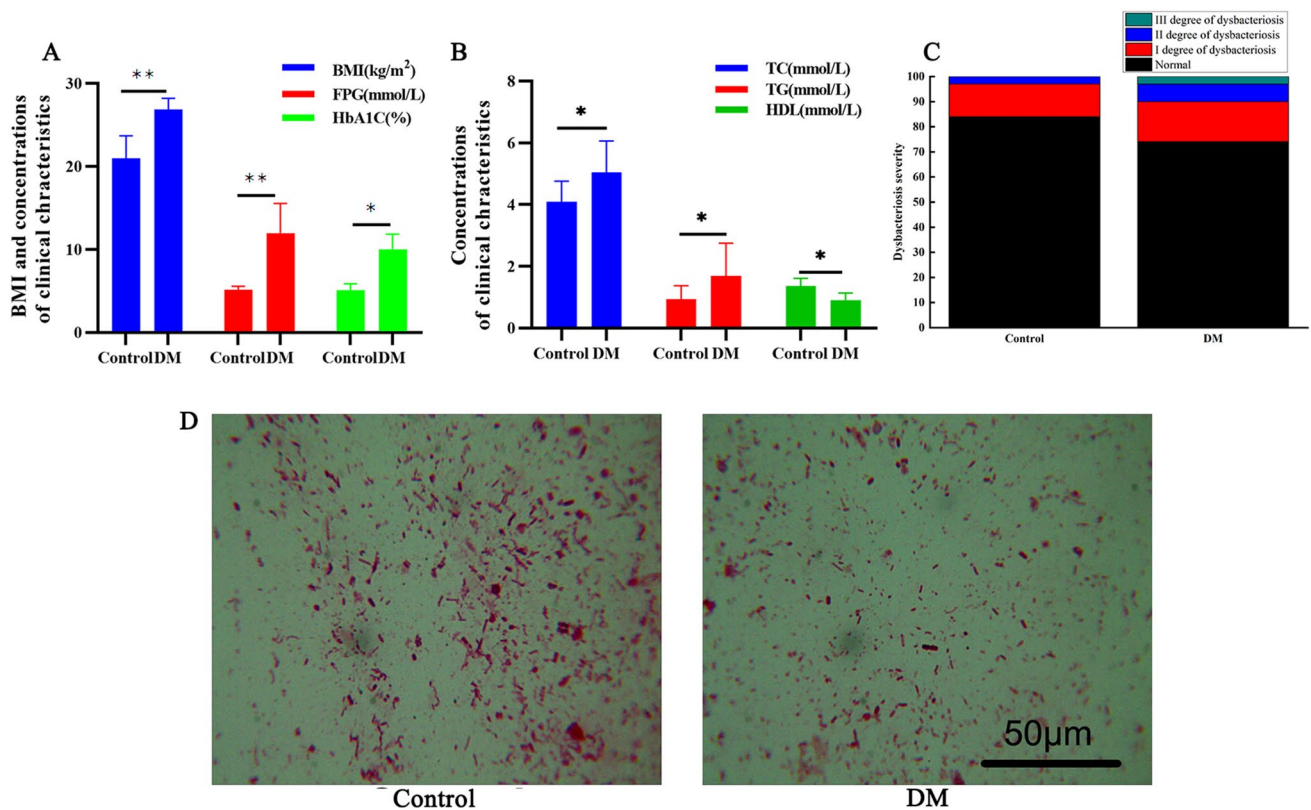
## Results

### Biochemical Analysis and Anthropometrics

As shown in Fig. 1A and B, compared with the control group ( $20.96 \pm 2.72$ ) kg/m<sup>2</sup>, the level of BMI in the

**Table 1** Biochemical analysis and anthropometrics

Parameter	Control (n = 16)	DM (n = 29)	P value
BMI (kg/m <sup>2</sup> )	20.96 $\pm$ 2.72	26.86 $\pm$ 1.34	$P < 0.01$
FPG (mmol/L)	5.17 $\pm$ 0.40	11.96 $\pm$ 3.57	$P < 0.01$
HbA1c (%)	5.09 $\pm$ 0.77	10.00 $\pm$ 1.83	$P < 0.01$
TC (mmol/L)	4.09 $\pm$ 0.68	5.04 $\pm$ 1.02	$P < 0.01$
TG (mmol/L)	0.94 $\pm$ 0.43	1.69 $\pm$ 1.06	$P < 0.05$
HDL (mmol/L)	1.37 $\pm$ 0.24	0.90 $\pm$ 0.24	$P < 0.01$



**Fig. 1** In comparison to the control group, T2DM patients had greater levels of BMI, FPG, HbA1c, TC, and TG, while their HDL concentration was lower. Results of microscopic examination by direct smear and microbiota dysbiosis analysis for stool. The abundance of intestinal microorganisms was dramatically reduced, and the degree of microbiota dysbiosis was increased in the T2DM group. **A:** the

analysis and comparison of BMI, FPG, and HbA1c between the control and T2DM groups. **B:** the analysis and comparison of TC, TG, and HDL between the control and T2DM groups. **C:** comparison of gut dysbacteriosis analysis. **D:** the original image of the fecal smears ( $\times 1000$ ). (mean  $\pm$  SE,  $n = 16$  in control group,  $n = 29$  in T2DM group,  $*P < 0.05$ , vs. control,  $**P < 0.01$ , vs. control)



T2DM group was higher ( $26.86 \pm 1.34$ ) kg/m<sup>2</sup> ( $P < 0.01$ ), the FPG was significantly higher in the T2DM group ( $11.96 \pm 3.57$ ) mmol/L than the control group ( $5.17 \pm 0.4$ ) mmol/L ( $P < 0.01$ ), and the HbA1c level in the T2DM group ( $10.00 \pm 1.8$ ) % was significantly higher than control group ( $5.09 \pm 0.7$ ) % ( $P < 0.05$ ). In addition, TC and TG were significantly different ( $P < 0.05$ ) between the two groups, but the HDL was significantly lower in the T2DM group [ $(1.37 \pm 0.24)$  mmol/L vs.  $(0.90 \pm 0.24)$  mmol/L,  $P < 0.05$ ] (Table 1).

### Microscopic Examination and Gut Microbiota Analysis

The extent of gut microbiota dysbiosis was compared between the T2DM group and the control group. Data were expressed as the proportion of patients with type I, II, and III dysbiosis to the total number of patients in each group. The number of people with type I, II, and III dysbiosis of the microbiota changed from 13%, 3%, and 0% of the control group to 16%, 7%, and 3% in the T2DM group (Fig. 1C). Compared with the control group, the abundance of intestinal microorganisms was significantly decreased in the T2DM group, especially *bacilli* (Fig. 1D).

### Comparison and Analysis of the Gut Microbiota Composition

Taxonomically, eleven phyla, namely, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Tenericutes*, *Saccharibacteria*, *Fusobacteria*, *Euryarchaeota*, *Synergistetes*, and Unclassified, were observed in the T2DM and control groups (Fig. 2A). The phylum *Firmicutes* was the most abundant in the two groups, followed by the phylum *Bacteroidetes*. *Firmicutes* (72.15 vs. 78.59%), and *Proteobacteria* (1.97 vs. 6.61%) were significantly increased, whereas *Bacteroidetes* (21.12 vs. 10.37%) were decreased in the T2DM group compared with the control group.

At class level, *Clostridia*, *Bacteroidia*, *Gammaproteobacteria*, *Negativicutes*, *Bacilli*, *Actinobacteria*, *Coriobacteriia*, and *Erysipelotrichia* were the most representative groups of the microbiota and accounted for 59.97%, 21.12%, 1.46%, 3.91%, 7.01%, 2.59%, 1.85%, and 1.25% of the total composition in the control group and 71.71%, 10.37%, 5.91%, 4.35%, 2.08%, 2.53%, 1.32%, and 0.44% in the T2DM group, respectively (Fig. 2B). *Clostridia* was most abundant in the two groups, increasing in the T2DM group, whereas *Bacilli* and *Bacteroidia* decreased.

*Clostridiales*, *Bacteroidales*, *Enterobacteriales*, *Selenomonadales*, and *Lactobacillales*, as the dominant groups of the microbiota, presented differences between the two

groups at the order level (Fig. 2C). The specific representation was as follows: *Clostridiales* (59.97% vs. 71.71%) and *Enterobacteriales* (1.46% vs. 5.91%) were more abundant, whereas *Bacteroides* (21.12% vs. 10.37%) and *Lactobacillales* (7.01% vs. 2.08%) were less abundant in the T2DM group than in the control group.

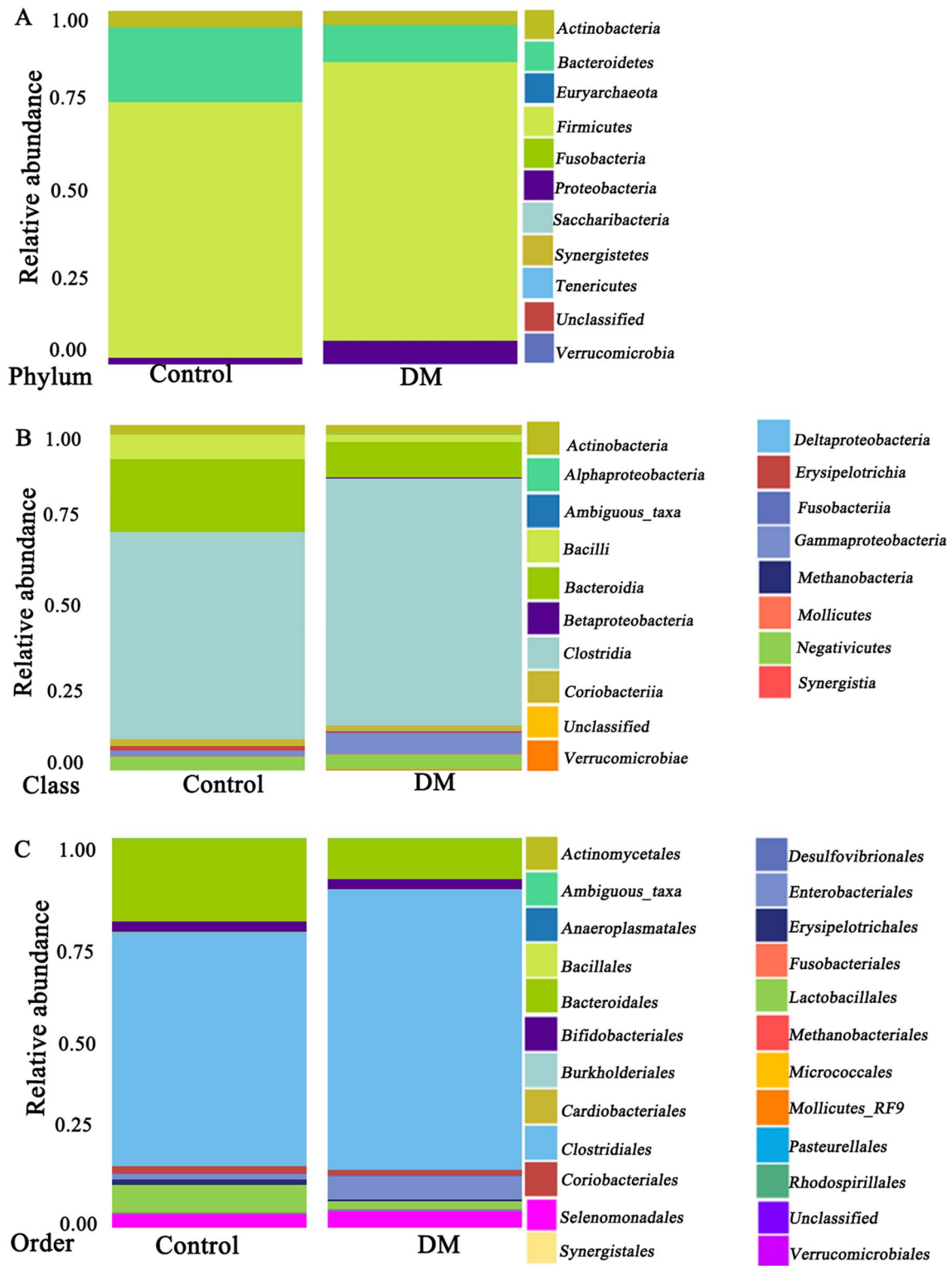
Forty OTUs were distributed across all samples at the family level, and *Ruminococcaceae* was the most abundant in the two groups, followed by *Lachnospiraceae* and *Bacteroidaceae* at the family level (Fig. 3A). In the T2DM group, we found that the relative abundances of *Enterobacteriaceae* and *Ruminococcaceae* increased by 4.45% and 12.71%, respectively, while those of *Bacteroidaceae*, *Lactobacillaceae*, and *Streptococcaceae* were reduced by 6.21%, 3.19%, and 1.68%, respectively, compared with the control group (Table 2).

*Faecalibacterium*, *Subdoligranulum*, *Bacteroides*, *[Eubacterium]\_cc\_rectale\_group*, *Escherichia-Shigella*, Unclassified, *[Eubacterium]\_coprostanoligenes\_group*, *Roseburia*, *Blautia*, *Dialister*, *[Ruminococcus]\_torques\_group*, *Bifidobacterium*, *Streptococcus*, *Prevotella\_9*, *Ruminococcaceae\_UCG-014*, *Ruminococcaceae\_UCG-002*, *Ruminococcaceae\_UCG-013*, *Dorea*, *Alistipes*, *Lactobacillus*, *Ruminococcus\_2*, *Christensenellaceae\_R-7\_group*, *Ambiguous\_taxa*, *Lachnospirillum*, *Clostridium\_sensu\_stricto\_1*, and *Alloprevotella* were the primary gut microbiota of the two groups at the genus level (> 1.0% of the total composition in all groups) (Fig. 3B). The relative abundances of *Bacteroides*, *Blautia*, and *Streptococcus* were decreased, whereas those of the *[Eubacterium]\_rectale\_group*, *Faecalibacterium*, and *Subdoligranulum* were increased in the T2DM group compared with the control group.

The intestinal microbiota mainly consisted of Unclassified (65.01 vs. 55.69%) and *Ambiguous\_taxa* (34.29 vs. 44.02%) at the species level, and the results showed that the abundance of *Ambiguous\_taxa* was increased by 9.73% in the T2DM group compared with the healthy group (Fig. 3C).

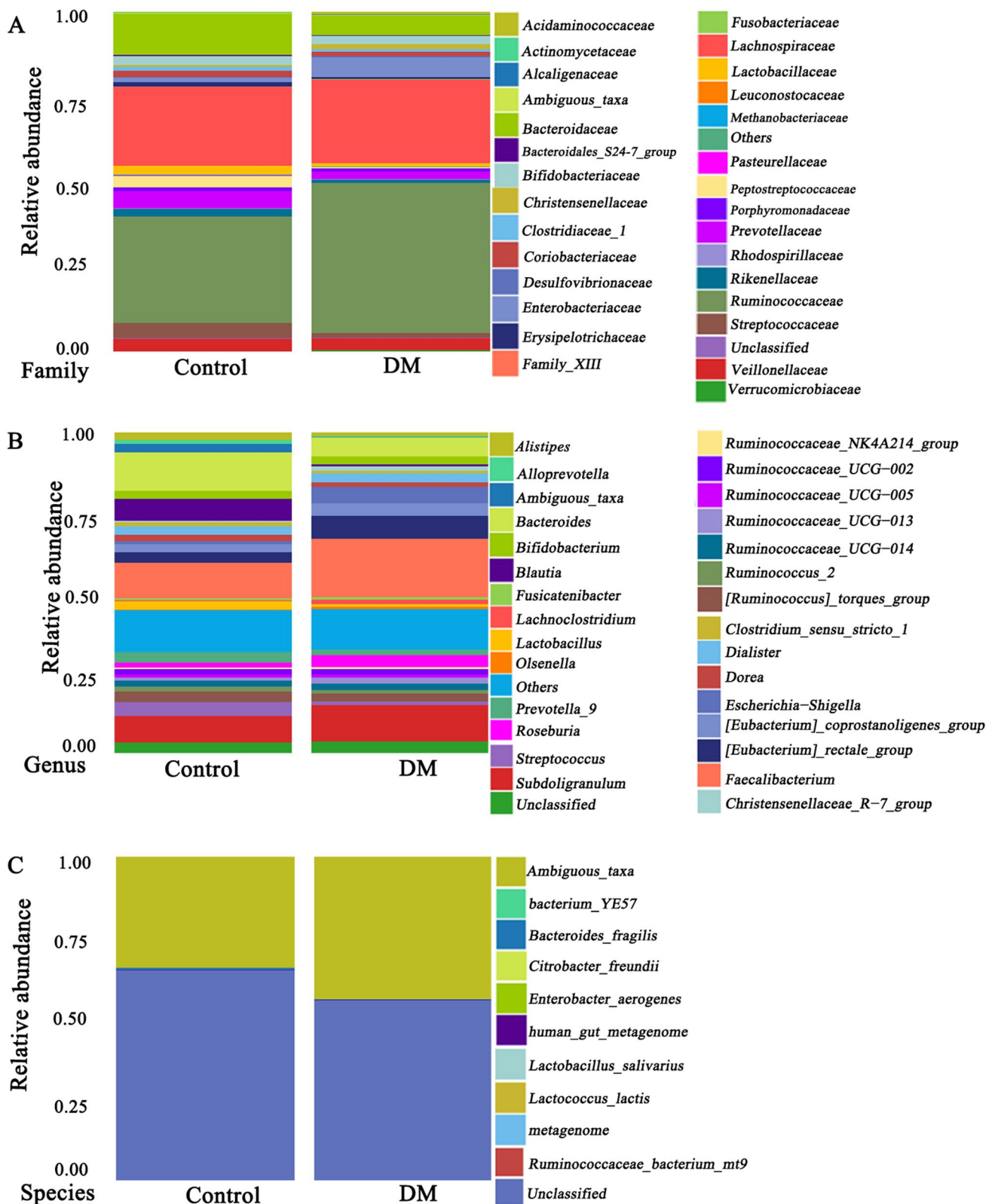
### Quantitative Analysis of Target Bacteria

Compared with the control group, the composition of the microbiota in the feces of T2DM patients changed significantly, mainly manifested as follows: several common bacteria such as *E.coli* in the feces of T2DM patients ( $1.15 \pm 0.12$ ) did not change compared with the control group ( $1.00 \pm 0.10$ ,  $P > 0.05$ ), while the mRNA expression of *Enterococci* was upregulated ( $0.10 \pm 0.01$  vs.  $1.80 \pm 0.13$ ,  $P < 0.05$ )



**Fig. 2** The relative abundances of the gut microbiota of all fecal samples at the phylum, class, order classification levels in the T2DM and control groups. **A:** the relative abundance of the major phyla. **B:** the

relative abundance of the major classes. **C:** the relative abundance of the major orders



**Fig. 3** The relative abundances of the gut microbiota of all fecal samples at the family, genus, and species classification levels in the T2DM and control groups. **A:** the relative abundance of the major

families. **B:** the relative abundance of the major genus. **C:** the relative abundance of the major species

(Fig. 4A), and the expression of *Bacteroides* ( $21.13 \pm 3.12$  vs.  $1.15 \pm 0.12$ ,  $P < 0.01$ ), *Bifidobacteria* ( $8.32 \pm 2.35$  vs.  $0.21 \pm 0.05$ ,  $P < 0.05$ ), and *Lactobacilli* ( $46.50 \pm 7.14$  vs.  $3.12 \pm 1.13$ ,  $P < 0.05$ ) was significantly downregulated compared to those in the healthy group (Fig. 4B).

### Comparison of the SCFA Concentrations

Fecal samples from the T2DM group and the control group were assessed for SCFA concentrations. The results were shown in Fig. 4C. The figure showed that the total SCFA concentrations of the T2DM group were lower than that of

the control group and that acetic acid and propionic acid were the most prominent ( $P < 0.01$ ).

### Comparison of the D-Lactic Acid Concentrations

The average concentration of D-lactic acid in the control group was ( $114.95 \pm 13.31$ ) nmol/mL. Compared with the control group, the D-lactic acid concentration in the T2DM group was significantly lower ( $88.86 \pm 6.87$ ) nmol/mL ( $p < 0.01$ ) (Fig. 4D).

### Correlation Analysis Between FPG, HbA1c, and Target Bacteria

As seen in Table 3, Pearson correlation analysis showed that FPG was positively correlated with *Enterococcus* ( $r = 0.839$ ,  $P = 0.009$ ) and negatively correlated with *Bifidobacteria* ( $r = -0.800$ ,  $P = 0.017$ ), *Bacteroides* ( $r = -0.822$ ,  $P = 0.012$ ), and *Lactobacillus* ( $r = -0.795$ ,  $P = 0.018$ ). HbA1c was negatively correlated with *Lactobacillus* ( $r = -0.734$ ,  $P = 0.038$ ), while there was no significant correlation with *Enterococcus*, *Bifidobacterium*, or *Bacteroides*.

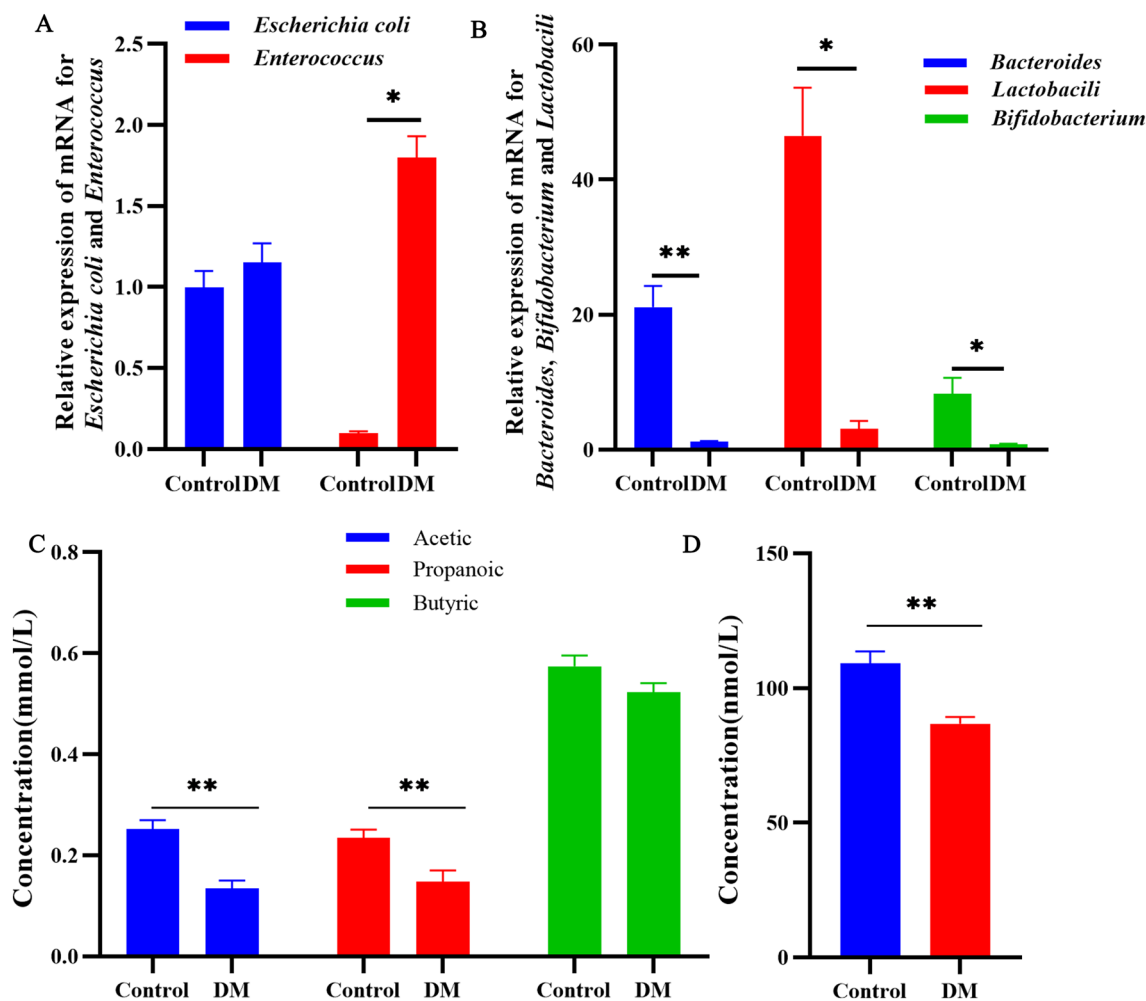
### Discussion

The effect of microbiota on glucose metabolism in both animal models of T2DM and clinical trials has demonstrated that microbiota is associated with the pathophysiology of T2DM. In preclinical animal models as well as in patients with type 2 diabetes and complications, there were differences in gut microbiota composition compared to healthy controls. The severity of gut microbiota dysbiosis correlated with disease severity, and recovery with probiotics in animal models and human patients was associated with improved symptoms and disease progression [10]. However, the results in humans have been less promising, with few and largely inconsistent clinical trials, and the literature on T2DM raises concerns about the variability of outcomes. To clarify the specific changes in the gut microbiota in patients with T2DM, we performed microscopic examination by direct smear and comparison analysis of species abundance and diversity of feces from T2DM patients and healthy people in this work. The results suggested that T2DM patients presented a lower diversity of gut microbiota and a more serious microbiota dysbiosis than healthy subjects. Similarly, a study conducted on the gut microbial DNA sequences from 345 Chinese individuals found that there was moderate intestinal dysbacteriosis in diabetic patients, and the abundance of butyric acid-producing bacteria that might be metabolically beneficial decreased, while several opportunistic pathogens increased [16].

**Table 2** The analysis of the diversity of microbiota

	Control	DM
Phylum		
<i>Firmicutes</i> (%)	78.59	72.15
<i>Proteobacteria</i> (%)	6.61	1.97
<i>Bacteroidetes</i> (%)	10.37	21.12
Class		
<i>Clostridia</i> (%)	59.97	71.71
<i>Bacteroidia</i> (%)	21.12	10.37
<i>Gammaproteobacteria</i> (%)	1.46	5.91
<i>Negativicutes</i> (%)	3.91	4.35
<i>Bacilli</i> (%)	7.01	2.08
<i>Actinobacteria</i> (%)	2.59	2.53
<i>Coriobacteriia</i> (%)	1.85	1.32
<i>Erysipelotrichia</i> (%)	1.25	0.44
Order		
<i>Clostridiales</i> (%)	71.71	59.97
<i>Enterobacteriales</i> (%)	5.91	1.46
<i>Bacteroides</i> (%)	10.37	21.12
<i>Lactobacillales</i> (%)	2.08	7.01
Family		
<i>Enterobacteriaceae</i> (%)	1.46	5.91
<i>Lachnospiraceae</i> (%)	23.26	24.82
<i>Ruminococcaceae</i> (%)	31.55	44.26
<i>Bacteroidaceae</i> (%)	12.03	5.82
<i>Lactobacillaceae</i> (%)	2.51	0.83
<i>Streptococcaceae</i> (%)	4.37	1.18
Genus		
<i>Bacteroides</i> (%)	12.04	5.82
<i>Blautia</i> (%)	6.78	0.67
<i>Streptococcus</i> (%)	4.37	1.17
<i>[Eubacterium]_rectale_group</i> (%)	3.20	7.18
<i>Faecalibacterium</i> (%)	11.10	18.35
<i>Subdoligranulum</i> (%)	8.17	11.30
<i>Lactobacillus</i>	2.51	0.83
Species		
Unclassified (%)	65.01	55.69
Ambiguous_taxa (%)	34.29	44.02





**Fig. 4** Quantitative analysis of real-time PCR of target bacteria in the T2DM and control groups. Compared with the control group, the quantities of microbiota in the feces of T2DM patients changed significantly. Comparison of the SCFA and D-lactic acid concentrations. The concentrations of SCFAs and D-lactic acid in the T2DM group were less than those in the control group, and acetic acid and propi-

onic acid were the most obvious. **A:** the relative abundance of *E. coli* and *Enterococcus*. **B:** the relative abundance of *Bacteroides*, *Lactobacilli*, and *Bifidobacterium*. **C:** SCFA content in the control group and T2DM group. **D:** D-Lactate concentrations in the control group and T2DM group (mean ± SE, n = 16 in control group, n = 29 in T2DM group. \*P < 0.05, vs. control, \*\*P < 0.01, vs. control)

**Table 3** Correlations between the level of FPG, HbA1c, and target bacteria

Blood glucose index	<i>Enterococcus</i>	<i>Bacteroides</i>	<i>Lactobacilli</i>	<i>Bifidobacterium</i>
FPG	r = 0.839 P = 0.009	r = -0.822 P = 0.012	r = -0.795 P = 0.018	r = -0.800 P = 0.017
HbA1c	r = 0.445 P = 0.269	r = -0.462 P = 0.249	r = -0.734 P = 0.038	r = -0.367 P = 0.371

Moreover, in our study, there was a significant difference in the intestinal microbiota composition between the two groups. At the phylum level, the T2DM group presented a preponderance of *Firmicutes*, followed by *Bacteroidetes*,

*Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*. The T2DM group had higher levels of *Firmicutes* and *Proteobacteria* but a lower level of *Bacteroidetes* than the control group. The increase in *Firmicutes/Bacteroidetes* (F/B) ratio is similar to the study by Krogius-Kurikka et al. who reported a higher F/B ratio in T2DM mice than in controls, and treatment with the probiotic *L. acidophilus* significantly moderated the F/B ratio by increasing the *Bacteroidetes* abundance [17]. Conversely, Larsen et al. found that the ratio of F/B decreased and was negatively correlated with FPG in the diabetes group. This issue was also discussed in the study of obese subjects [18]. An increase in the *Clostridia* class and a decrease in *Bacteroidia* class have been observed in T2DM [19], which is consistent with our findings.

A positive correlation between *Ruminococcaceae* with T2DM has been reported; however, the results are inconsistent. Different types of treatment may be a major reason for the inconsistent results of these studies [7]. Our data showed that *Ruminococcaceae*, *Lachnospiraceae*, and *Bacteroidaceae* were enriched in the two groups, and OTUs identified as *Enterobacteriaceae* and *Ruminococcaceae* had relatively high abundances in the T2DM group at the family level.

Three other researches found a positive correlation between *Bacteroides* and disease, in contrast to the five cross-sectional studies that found a negative correlation. In studies on animals, the treatment of *Bacteroides acidifaciens* and *Bacteroides uniformis* reduced IR and improved glucose intolerance in diabetic mice [7]. *Lactobacillus* showed some protective effects against T2DM, the majority combined with other genera, most frequently *Bifidobacterium* [20]. In our study, *Bacteroides*, *Blautia*, *Prevotella*, *Lactobacillus*, and *Streptococcus* were decreased, whereas [*Eubacterium*]*\_rectale\_group*, *Faecalibacterium*, and *Subdoligranulum* were increased in the T2DM group. *Prevotella* was shown to be negatively correlated with the levels of LPS and FPG [21], which was consistent with our study.

LPS, produced from the gram-negative bacterial cell wall, can initiate metabolic endotoxemia by combining with toll-like receptor 4 (TLR4), which can increase the concentration of pro-inflammatory cytokines, such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6. The administration of LPS could induce weight gain, high levels of FPG, and aggravated IR in T2DM mice [22]. The increased abundance of *Enterococcus*, a gram-negative bacterium in our paper, played a positive role in the development of T2DM by promoting the production of LPS. *Bacteroidetes* have beneficial effects on glucose metabolism in humans and experimental animals, which are negatively correlated with disease [7], and the abundance of *Bacteroides* was significantly reduced in our study, validating the conclusions of previous studies. Cross-sectional studies of patients versus controls reported a positive association between the abundance of the *Lactobacillus* genus and T2DM [22]. It is suggested that *Lactobacillus* and *Bifidobacterium* may work synergistically [23]. *Bifidobacterium* appears to be the genus that is most consistently supported by the literature as containing microbes that are potentially protective against T2DM and show improvement in glucose tolerance [7, 24]. In our study, the relative abundances of *Bacteroides*, *Bifidobacteria*, and *Lactobacillus* were significantly reduced. High-throughput sequencing was performed and found that there was a significant difference between the two groups in the abundance of *Enterococcus*, *Bacteroides*, and *Lactobacillus* but not in *Bifidobacteria*, which may be due to the relatively small sample size or different analysis methods used in this work.

SCFAs have been shown to play multiple roles in IR and T2DM, including promoting the integrity of the intestinal epithelium, controlling immunomodulatory functions, and regulating islet  $\beta$ -cell proliferation and insulin secretion [25]. SCFA-producing bacteria, such as *Clostridiales*, *Ruminococcaceae*, *Prevotella*, *Bacteroides*, *Lactobacillus*, and *Blautia*, can produce SCFAs that have antiobesity and antidiabetic effects, while *Clostridiales*, *Coprococcus*, and *Lachnospiraceae* can inhibit the production of SCFAs [17, 26]. In our paper, the T2DM group presented a decreased abundance of *Bacteroides*, *Lactobacillus*, *Prevotella*, and *Blautia*, accompanied by decreased acetic acid, propanoic acid, and butyric acid, which may account for the increased plasma glucose level.

Ding et al. proved that *Prevotella* and *Bacteroides* were negatively correlated with blood glucose levels, and *Lactobacillaceae*, *Ruminococcaceae*, and *Veillonellaceae* were significantly positively correlated with FPG [26], but some controversy remains. Several cross-sectional studies of patients versus controls reported a positive association between the abundance of this genus and T2DM [27, 28]. For instance, Karlsson et al. found that the richness of *Lactobacilli* increased in diabetic patients compared with the control group, and *Lactobacilli* was positively correlated with FPG [29]. Many studies have shown that *Lactobacillus Plantarum* improves glucose metabolism in diet-induced and genetic models of T2DM mice [30–33]; however, in four clinical trials, this species had no significant effect on glucose metabolism [34–37]. This result may be attributed to the fact that the *Lactobacillus* genus is highly diverse, and its effect on T2DM appears to be species- or even strain-specific, which may explain the lack of consistency in genus-level analyses among studies using this microbiota [7]. Our data showed that FPG was positively correlated with *Enterococcus* but negatively correlated with *Bifidobacterium*, *Bacteroides*, and *Lactobacillus*, and the HbA1c level was negatively correlated with *Lactobacillus*.

There were some limitations in this paper, such as the general condition of the patients, the course of the disease, that could not meet the unified standard. In addition, the microbiota was detected from the host feces sample, rather than directly measured from the intestinal mucosa. The microbial equivalence between the two methods needs more scientific evidence for support. We only paid attention to the common bacteria in this study, so more and deeper related research is expected to be conducted in future.

## Conclusion

There are obvious changes in the microbiota in patients with T2DM, and these changes are closely related to the level of plasma glucose in patients with diabetes. These findings

revealed that gut microbiota plays an important role in the pathogenesis of T2DM. FPG and HbA1c, as important indices to assess the condition of diabetic patients, have been identified to be correlated with some target bacteria, which may provide an auxiliary reference for judging the prognosis of patients. Considering that alterations in the microbiota can affect glucose metabolism, micro-ecological treatment is a potential intervened method to impede the progression of diabetes mellitus via the improvement in intestinal microbiota structure. Admittedly, this issue remains controversial regarding the complexity and diversity of gut microbiota. Thus, more reasonable and strict clinical tests should be carried out in the future to elucidate the changes in the gut microbiota in T2DM patients.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00284-023-03219-x>.

**Acknowledgements** The authors are grateful for Xuhong Lin's foundation and great technical support.

**Author Contributions** XHL and QLH designed the study. XHL, QLH, HCW, YKM, RLY, ZFD, JNY, YX, DDW, and LPZ collected the samples and clinical information and analyzed the data. QLH, HCW, YKM, RLY, ZFD, and JNY analyzed and interpreted the data. XHL and QLH wrote and revised the manuscript. All authors had full access to the final version of the report and agreed to the submission.

**Funding** This work was supported by projects supported by the National Natural Science Foundation of China (No. 81500430 and No. U1304802), the Key Scientific Research Projects of Higher Education in Henan (No. 17A320019), and the Henan Science and Technology Planning Project (No. 182102310544 and No. 192102310045).

**Data Availability** The sequencing data have been deposited in the NCBI SRA database (<http://www.ncbi.nlm.nih.gov/SRA/>), BioProject accession PRJNA89876. All other data are included within the article or available from the authors upon request.

**Code Availability** Not applicable.

## Declarations

**Conflict of interest** The authors have no conflict of interest to declare.

**Ethical Approval** All human protocols of this study were approved by the ethics committee of Huaihe Hospital, Henan University, where the work was conducted, and this study complies with the Declaration of Helsinki with ethics approval number 2017111.

**Consent to Participate** Written and verbal informed consent were obtained from all participants.

**Consent for Publication** All participants provided consent to publish the results.

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