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ORIGINAL ARTICLE

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Comprehensive relationships between gut microbiome and faecal metabolome in individuals with type 2 diabetes and its complications

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Abstract

Purpose As the treatment regimens such as metformin could confound the correlation between type 2 diabetes (T2D) and gut microbiome, we should revisit the relationship between gut microbiota and T2D patients who are not currently treated with metformin.

Methods The study recruited 65 T2D patients: 49 with and 16 without diabetic complications, and 35 healthy controls. We sequenced the 16S rRNA V3-V4 region of gut microbiota and detected metabolites based on liquid chromatography mass spectrometry (LC/MS) and gas chromatography mass spectrometry (GC/MS) in faecal samples.

Results The composition of both the gut microbiota and faecal metabolites changed significantly with T2D patients. The abundance of *Proteobacteria* and the ratio of *Firmicutes/Bacteroidetes* were higher in T2D patients than healthy subjects, and the short chain fatty acids (SCFAs), bile acids and lipids of T2D patients were significantly disordered. Moreover, the abundances of certain SCFA-producing bacteria (*Lachnospiraceae* and *Ruminococcaceae etc*.) were significantly increased in T2D patients, while the faecal SCFAs concentrations were significantly decreased. It's suggested that the role of SCFA-producing bacteria was not simply to produce SCFAs. Then we identified 44 microbial modules to explore the correlations between the gut microbiota and metabolic traits. Specially, most modules including certain SCFA-producing bacteria were comprehensively correlated to body mass index, the levels of blood glucose, blood pressure, blood cholesterol and faecal bile acids and lipids.

Conclusions Our study identified the relationships between the gut microbiota and faecal metabolites, and provided a resource for future studies to understand host–gut microbiota interactions in T2D.

Keywords Gut microbiota · Faecal metabolites · Short chain fatty acid · Correlation · Type 2 diabetes

Introduction

Recent studies have shown that the gut microbiome is closely related to various metabolic diseases, such as type 2 diabetes (T2D) [1]. However, several associations between gut microbial dysbiosis and T2D differed between studies [2, 3]. One of the reasons is that the treatment regimens such as metformin could alter the composition and function of gut microbiota [4]. It is meaningful to explore the composition of the gut microbiota in T2D patients who are not taking metformin.

Multiple studies have established that microbial metabolites have a major influence on host physiology [5, 6]. The most commonly known fermentation products are short chain fatty acids (SCFAs), especially acetate, propionate and butyrate, which are not only energy sources and

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signalling molecules in the gut, but might also enter the systemic circulation and directly affect metabolism or function of the peripheral tissues [7]. Another example is trimethylamine *N*-oxide (TMAO), which is derived from phosphatidylcholine (PC) by gut microbiota, has been confirmed to play an important role in atherosclerosis [8]. In addition, branched-chain amino acids [9, 10], aromatic amino acids [11], glutamate [12] and amino acid-derived metabolites [13] have been reported as microbially modulated metabolites, which are harmful to metabolic diseases. Since the gut microbiota can affect the metabolic process of host physiology through its related products, the interplay between gut microbiota and faecal endogenous metabolites might also mediate the associations between T2D and gut microbiota.

In the study, we enroled a cohort of one hundred 40–60 years old Chinese individuals including 65 T2D patients without metformin or Traditional Chinese Medicine (TCM) treatment and 35 healthy controls to further analyse the gut microbiota and metabolites in their faecal samples. The differential gut microbiota and faecal metabolites in the T2D patients compared with the healthy subjects were examined. In addition, the functional pathways based on gut microbiota and faecal metabolites were predicted. We also identified some correlations between gut microbiota and clinical characteristics/faecal metabolites in T2D patients and healthy subjects. Finally, we focused on the interactions in some SCFA-producing bacteria and SCFAs, bile acids and PCs, which might provide resources for future studies to understand host–gut microbiota interactions in T2D.

Materials and methods

Subjects

We consecutively enrolled 65 T2D patients who were hospitalised for hyperglycaemia in the Department of Endocrinology of the Second Hospital of Shandong University. All T2D cases were diagnosed by having a fasting blood glucose (FBG) of ≥7.0 mmol L^{-1}, post-oral glucose tolerance test (OGTT) of ≥11.1 mmol L^{-1} or a HbA_{1c} of ≥ 6.5% [14]. The T2D cases (T2D group, $n = 65$) were further divided into two subgroups: T2D with chronic complications (T2D+ group, $n = 49$) and T2D without chronic complications (T2D− group, $n = 16$). The T2D individuals with chronic complications were registered by two professional diabetologist in the morning the day after admission. Briefly, a fundus camera was used to obtain retinal photography and screen for retinopathy; 10-g monofilament testing, 128-Hz tuning fork testing, tendon reflexes or electrophysiological testing were used to screen diabetic peripheral neuropathy; A systolic blood pressure (SBP) ≥ 140 mmHg or a diastolic blood pressure (DBP) ≥ 90 mmHg was defined as hypertension; and whether or not patients had established cardiovascular disease was noted. T2D− group was defined as newly diagnosed T2D without complications. Thirty-five age- and sex-matched healthy controls with FBG < 6.1 mmol L^{-1} and HbA_{1c} of < 6.5% [15], and without metabolic syndrome (e.g. having a body mass index ≥ 24 kg m^{2-1}, hypertension or dyslipidemia etc.) were recruited from volunteers at the Physical Examination Centre of the Second Hospital of Shandong University. The patients in T2D+ group received therapies for hyperglycaemia, hypertension and/or dyslipidemia by insulin, aspirin, irbesartan, valsartan, atorvastatin or fenofibrate, but not by metformin or TCM. The subjects in T2D− and healthy groups were not received concomitant therapies including drugs, health care products, probiotics or its products. The exclusion criteria for all research subjects included constipation or diarrhoea, hepatitis, an alcohol intake of more than 20 g day^{-1}, smoking, or an intake of probiotics or antibiotics for more than 3 days in the previous 8 weeks. The baseline clinical characteristics of all subjects are summarised in Table 1.

Informed consent was obtained from all subjects, the study was performed under the guidance of the Helsinki Declaration, and was approved by the Committee on the Ethics of the Second Hospital of Shandong University. The study was registered in the Research Chinese Clinical Trial Registry (No. ChiCTR1800014700).

Sample collection, preparation, sequencing and data processing

Fasting faeces were aseptically collected, immediately frozen at −20 °C, then stored at −80 °C within 24 h until analysis.

Gut microbial DNA was extracted from a 0.2 g thawed faecal sample using a FastDNA® Spin Kit for soil (MP Biomedical, LLC, catalogue 116560-200) following manufacturer instructions. The 16s rRNA V3-V4 region was amplified by Primer F (5'- AACGGGAAGACAACGT ACGG -3') and Primer R (5'- CAGATGCAGGAGGACAT GTC -3') with barcode sequence. Library was constructed following the manufacturer's instructions of the Ion Plus Fragment Library Kit and sequenced by Ion $S5^{TM}$ Sequencer. The 16S raw sequencing reads are available in the NCBI Sequence Read Archive (SRA) database under the SRA accession number SRP168691.

The raw reads were de-multiplexed using USEARCH software [16]. Operational taxonomic unit (OTU) picking was conducted using the QIIME [17]. 16S rRNA gene sequences were clustered at a similarity cutoff value of 97% using UCLUST [16]. Matching of OTUs to bacteria was then conducted using the SILVA reference database [17]. Microbial composition at each taxonomic level was defined using the summarise taxa function in QIIME. Alpha and

Table 1 Clinical and biological characteristics of type 2 diabetes and healthy subjects

	Healthy	T2D	T2D−	T2D+
Gender (male/female)	35 (18/17)	65 (35/30)	16 (7/9)	49 (28/21)
Age (years)[a]	44 (42, 48)	52 (43, 58)	52 (40, 58)	52 (44, 58)
BMI (kg $m^{2\ -1}$)[b]	22.01 ± 1.32	25.20 ± 3.84**	23.33 ± 3.92	25.81 ± 3.64**
WC (cm)[a]	74 (70, 81)	87 (81, 99)**	83 (80, 90)*	90 (84, 100)**
HC (cm)[a]	91 (88, 97)	100 (94, 105)**	96 (93, 99)	101 (97, 106)**
WHR (WC/HC)[a]	0.81 (0.79, 0.84)	0.89 (0.86, 0.94)**	0.89 (0.87, 0.90)**	0.90 (0.86, 0.95)**
SBP (mmHg)[b]	117.03 ± 8.70	141.22 ± 20.77**##	126.06 ± 10.12*	146.16 ± 21.02**##
DBP (mmHg)[b]	72.69 ± 7.76	86.38 ± 11.04**#	80.56 ± 9.52*	88.29 ± 10.92**##
HbA_{1c} (%)[b]	4.38 ± 0.78	9.42 ± 2.49**	9.65 ± 2.65**	9.35 ± 2.47**
FBG (mmol L^{-1})[a]	5.33 (4.06, 5.15)	9.55 (6.72, 11.96)**	9.55 (7.74, 12.23)**	9.55 (6.53, 11.96)**
TC (mmol L^{-1})[a]	4.82 (4.35, 5.03)	5.08 (4.33, 5.76)	5.10 (4.23, 5.68)	5.08 (4.35, 6.55)
TG (mmol L^{-1})[a]	1.06 (0.84, 1.34)	1.29 (0.93, 1.75)*	1.48 (1.21, 1.98)*	1.16 (0.93, 1.70)
LDLC (mmol L^{-1})[b]	2.33 ± 0.57	2.70 ± 0.90	2.74 ± 0.71	2.69 ± 0.97
HDLC (mmol L^{-1})[a]	1.42 (1.13, 1.60)	1.24 (1.03, 1.49)	1.76 (1.09, 1.25)	1.31 (0.99, 1.58)

BMI body mass index, *WC* waist circumference, *HC* hip circumference waist, *WHR* waist-hip ratio, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *FBG* fasting blood glucose, *HbA1c* glycated haemoglobin, *TC* total cholesterol, *TG* triacylglycerol, *LDLC* low-density lipoprotein cholesterol, *HDLC* high-density lipoprotein cholesterol, *Healthy* healthy control, *T2D−* T2D patients without complications, *T2D+* T2D patients with complications, *T2D* T2D− and T2D+ patients

*$P < 0.05$; **$P < 0.01$; compared to Healthy group

#$P < 0.05$; ##$P < 0.01$; compared to T2D– group

[a]Median (25th, 75th) for non-distributed variables or percentages (%). *P* values were calculated by using Kruskal-Wallis *H*-test with Bonferroni adjustment

[b]Mean ± standard deviation for normally distributed variables. *P* values were calculated by using one-way analysis of variance (ANOVA) followed by LSD test or Dunnett's test

beta diversity plots were also generated using QIIME, two-sided student's two-sample *t*-test with Bonferroni correction was used to compare the unweighted UniFrac distances within or between groups [18].

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis

For functional metagenome prediction, 97% of the OTUs were picked using a closed-reference OTU picking protocol (QIIME) and the Greengenes database [19]. Reconstruction of the metagenome was performed using PICRUSt [20]. Predicted functional genes were catalogued into Kyoto Encyclopedia of Genes and Genome orthology (KO), and compared between research groups using STAMP [21].

Faecal SCFAs and endogenous metabolites measurements

The faecal SCFAs, including acetate, propionate, butyrate, isobutyrate, valeric acid, isovaleric acid and hexanoic acid were measured using Agilent 7890A equipped with an 7000D Triple Quadrupole gas chromatography mass spectrometry System (Agilent, USA), fitted with a HP-FFAP column (30 m × 0.25 mm × 0.25 μm, Agilent, USA). The standard solutions and faecal sample (0.2 g) were prepared as previously described [22]. The chromatography was programmed for an initial temperature of 100 °C for 1 min. The temperature was increased to 145 at 5 °C min^{-1}, increased to 240 at 40 °C min^{-1}, held for 10 min. Helium was used as the carrier gas (flow rate: 1 mL min^{-1}). The mass spectrometer was operated in the electron impact (70 ev) and selected ion monitoring (SIM) mode both for qualitative and for quantitative analysis with a solvent delay of 3 min. The typical total ion chromatogram of SCFAs standards and faecal samples are described in Supplementary Fig. S1. The SCFAs concentration were calculated by the standard linear regression curves [23].

An ACQUITY UPLC I-Class system & VION IMS QTOF Mass spectrometer (Waters, USA) was used to analyse endogenous faecal metabolites. 0.2 g of thawed faeces was precipitated with 5 mL methanol containing 1 μg mL^{-1} L-chlorophenylalanine. Samples were vortex mixed for 1 min. After 60 min of incubation at 4 °C, samples were stored overnight at −20 °C to improve protein

precipitation and then centrifuged at 14 000 × g for 20 min at 4 °C [24]. 1 µL of supernatant was injected into an ACQUITY UPLC BEH C18 column (100 × 2.1 mm, 1.7 µm, Waters, USA) maintained at 45 °C. A quality control (QC) sample, which was prepared by mixing equal volumes (50 µL) of each faecal sample supernatant, was injected after every 10th faecal samples to monitor system stability. The mobile phase was composed of formic acid/water (1/1000, v/v, A) and formic acid/acetonitrile (1/1000, v/v, B), the linear gradient conditions were 5–20% B (0–1.0 min), 20–40% B (1.0–2.5 min), 40–100% B (2.5–9.0 min), 100% B (9.0–12.0 min), 100–5% B (12.0–12.5 min) and 5% B (12.5–14.5 min), the flow rate was 0.4 mL min^{-1}. The Vion IMS QTOF MS specific conditions were set as follows: the lockspray ion source was operated in positive and negative electrospray ionisation mode (ESI), and the scan range was set from 50 to 1000 amu. The MS^E mode was used to collect MS data, as this scan mode includes a low-energy scan (CE 4 eV) and a high-energy scan (CE ramp 20–45 eV), the typical base peak ion chromatogram of each group is described in Supplementary Fig. S2. The raw data were imported to Progenesis QI (Waters, USA) for peak alignment to obtain a matrix including indices (retention time_m/z pairs), ion intensities and sample names. The matrix was further reduced by removing peaks with missing values in more than 80% samples. The repeatability of metabolomics data sets was assessed by the coefficient of variation in the QC samples at a threshold of 30% [25]. Then, the matrix was transferred to SIMCA-P 13.0 software (Umetrics, Sweden) for further statistical analysis. Partial least squares discrimination analysis (PLS-DA) was performed to globally analyse the metabolite differences between T2D and healthy groups. The differential metabolites were identified as variable of importance based on a projection (VIP value) > 1.0 from the seven-fold cross-validated orthogonal partial least squares discriminant analysis (OPLS-DA) model. These were validated by using Kruskal–Wallis *H*-test with Bonferroni adjustment and were further adjusted for BMI and age by multivariate analysis of co-variance. The online HMDB [26], LIPIDMAPS [27] and METLIN [28] databases were used to align the molecular mass data (m/z) and identify the differential metabolites. The pathway enrichment module of MetaboAnalysis was performed to analysis metabolic pathways [29].

Co-occurrence network analysis

In order to detect relationships between the gut microbiota and their potential combined effects on metabolic output, we constructed a network of co-occurrence taxa and interrogated the network for modules using weighted gene co-expression network analysis (WGCNA) [30]. Functionally related taxa were observed to cluster into the same modules preferentially, and the formation of modules depended upon additional ecological affinities, reflecting complementary convergent functionality [5]. Briefly, WGCNA constructs networks using the correlation coefficient between all possible pairs of the variable microbiota taxa as the co-expression measure, which was raised to a soft thresholding power (optimal beta = 12) to create the adjacency matrix. The topological overlap distance calculated from the adjacency matrix was then clustered with the average linkage hierarchical clustering, and modules were identified on the dendrogram using the Dynamic Tree Cut algorithm [31]. The correlation coefficient (absolute $r > 0.4$) between microbial modules and metabolic traits were used to explore the correlation of Hub-microbiota (eigengene connectivity value > 0.8) in modules and clinical characteristics/faecal metabolites. The significance of the correlations was determined by a Student asymptotic *P* value [5].

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software (IBM, USA). Shapiro–Wilk test was used to check the normality distributions of metabolic parameters. To analyse the statistical differences among the groups, normally distributed variables were assessed with one-way analysis of variance (ANOVA) followed by LSD test or Dunnett's test. Kruskal–Wallis *H*-test with Bonferroni adjustment was used to compare the not-normally distributed variables. Differential abundance of gut microbiota between T2D and healthy groups were tested by Welch's *t*-test, *P* values were corrected by Bonferroni correction for multiple tests. Statistical analyses were adjusted for BMI and age by multivariate analysis of covariance. The *post hoc* type on GPower 3.1 was used to calculate sample size and power. Box plots were performed using GraphPad Prime 7.0. The correlation network was visualised by Cytoscape 3.6.1. A P value < 0.05 was considered statistically significant.

Results

Clinical characteristics of T2D patients and healthy controls

The clinical characteristics of T2D and healthy groups are shown in Table 1. Compared with the healthy group, BMI, waist circumference (WC), hip circumference (HC), waist-hip ratio (WHR), HbA_{1c}, FBG, DBP, SBP and triacylglycerol (TG) were all significantly higher in the T2D group (one-way ANOVA or Kruskal–Wallis *H*-test, $P < 0.05$ or

0.01). In addition, SBP and DBP in the T2D+ group were significantly higher than the T2D− group (one-way ANOVA, $P < 0.01$).

Differences of gut microbial composition between T2D patients and healthy subjects

The raw gut microbial sequence data of 16S rRNA V3-V4 region were sorted into 6933 OTUs (>97% identity), and which were assigned to 526 taxa, with 245 of these presented in at least 80% of all samples. Overall, comparing the healthy group, the gut microbial richness was non-significantly higher in the T2D− group, but lower in T2D+ group. When T2D+ group was compared with T2D− group, the observed OTUs and chao index were significantly decreased (one-way ANOVA, $P < 0.05$, BMI and age adjusted $P < 0.05$) (Supplementary Fig. S3a and Table S1). For beta diversity, although unweighted unifrac distance-based principal coordinate analysis (PCoA) of the gut microbiome were not significantly different between the healthy group and T2D groups, most of the differences between groups were more significant than the differences within groups (student's *t*-test, $P < 0.001$) (Supplementary Fig. S3b, c), indicating that there were statistical differences between the three groups.

Welch's *t*-test was used to investigate the differences in gut microbial composition among the T2D and healthy groups. At phylum level, the most dominant microbial taxa in the three groups were *Firmicutes* (mean = 47.5%), *Bacteroidetes* (mean = 39.3%) and *Proteobacteria* (mean = 6.9%) (Supplementary Fig. S3d). *Bacteroides* (mean = 20.7%), *Prevotella* (mean = 10.1%), *Dialister* (mean = 7.6%), *Megamonas* (mean = 6.2%), *Ruminococcus* (mean = 4.7%), *Subdoligranulum* (mean = 4.7%), *Alistipes* (mean = 4.2%), *Fusobacterium* (mean = 3.9%), *Faecalibacterium* (mean = 3.7%), *Escherichia-Shigella* (mean = 2.6%), *Bifidobacterium* (mean = 2.3%) and *Lachnoclostridium* (mean = 1.8%) were the most highly abundant microbial taxa at genus level (Supplementary Fig. S3e).

Compared with the healthy group, the abundance of phylum *Firmicutes* and *Proteobacteria* were significantly increased in T2D group (Welch's *t*-test, $P < 0.001$), while *Bacteroidetes* was decreased (Welch's *t*-test, $P < 0.001$), and the ratio of *Firmicutes*/*Bacteroidetes* (F/B ratio) in T2D/T2D+ groups were higher than that in the healthy group. The *Actinobacteria* and *Tenericutes* were also significantly altered among the T2D groups and healthy group (Fig. 1a). At the taxonomic level (Fig. 1b), the *Bacteroides* and *Prevotella* from phylum *Bacteroidetes* in all T2D groups were significantly lower than those in the healthy group (fold change = −6.53 to −2.01, Welch's *t*-test, $P < 0.01$–0.05). The genus *Coprococcus 1* was significantly increased in T2D groups compared with the healthy group (fold change = 2.54–4.79, Welch's *t*-test, $P < 0.01$–0.02). Interestingly, the genera *Blautia* and *[Eubacterium] hallii group* were only significantly higher in the T2D− group than in the healthy group (fold change = 4.94 and 2.38, Welch's *t*-test, $P < 0.01$). Genus *Parasutterella* from *Proteobacteria* were also only significantly increased in the T2D+ group than in the healthy group (fold change = 7.49, Welch's *t*-test, $P < 0.05$).

When the T2D+ group was compared with the T2D− group, the ratio of *Firmicutes*/*Bacteroidetes* was increased, and the phylum *Firmicutes* was decreased, while the *Proteobacteria* was increased in the T2D+ group, that was reflected in genera *[Eubacterium] hallii group*, *Blautia*, *Coprococcus 1* and *Parasutterella*.

After adjusting for BMI and age, the statistical differences of certain microbiota in the phylum *Firmicutes* were changed when the T2D+ group compared with other groups (Supplementary Tables S2 and S3). Sample size and power calculation using differential gut microbiota as outcome variables showed that most of the power values were greater than 0.8 (Supplementary Table S4).

PICRUSt predicted analysis identified that carbohydrates, energy and lipids were significantly altered among T2D patients and healthy subjects. As shown in Fig. 1c, the carbohydrate metabolism, including TCA cycle and sugar metabolism were significantly depleted, and the glycerophospholipid metabolism, synthesis and degradation of ketone bodies and fatty acid metabolism were significantly enriched in T2D patient group.

SCFA differences in T2D patients and healthy subject

The faecal SCFAs were analysed by GC/MS. The standard linear regression curves and concentration of SCFAs are shown in Supplementary Tables S5 and S6. Compared with the healthy subjects, the concentrations of SCFAs including acetate, propionate, butyrate, valeric acid and hexanoic acid were predominantly reduced in the T2D patients (Kruskal–Wallis *H*-test, $P < 0.01$; BMI and age adjusted $P < 0.01$). However, these trends were not significant in the T2D− group (Fig. 1d), which indicated that the differences in SCFAs between T2D patients and healthy controls were mainly from the T2D+ group. When the T2D+ group was compared with the T2D− group, the concentrations of acetate, propionate and butyrate were significantly decreased (Kruskal–Wallis *H*-test, $P < 0.05$; BMI and age adjusted $P < 0.05$). Sample size and power calculation using differential faecal SCFAs as outcome variables showed that most of the power values were greater than 0.8 (Supplementary Table S8).

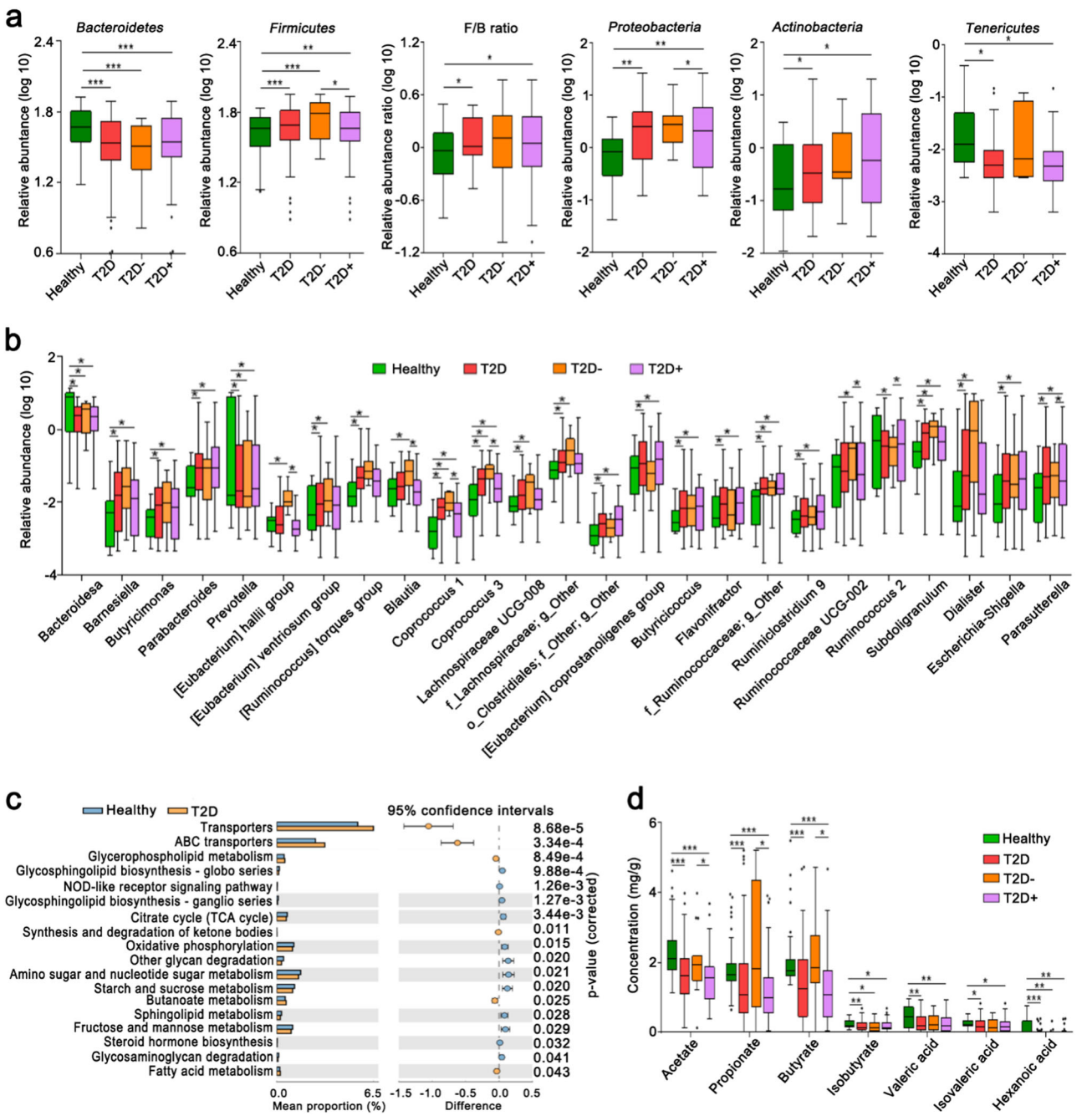

Fig. 1 Relative abundance of selected phyla **a** and genera **b** with significant differences among T2D and healthy groups. Welch's *t*-test corrected $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. F/B ratio, the ratio of Firmicutes/Bacteroidetes. Functional differences of gut microbiota in T2D and healthy groups **c**. The box plots of short chain fatty acids (SCFAs) in faecal samples of T2D and healthy groups **d**. Kruskal–Wallis *H*-test, Bonferroni adjusted $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0{\cdot}001$. Healthy healthy controls ($n = 35$), T2D− T2D patients without complications ($n = 16$), T2D+ T2D patients with complications ($n = 49$), T2D T2D− and T2D+ patients ($n = 65$)

Faecal metabolite profiles in T2D patients and healthy subjects

After LC/MS analysis of faecal samples, the metabolite profiles from the T2D and healthy groups were discriminative in PLS-DA patterns of positive and negative models (Fig. 2a). Moreover, individuals in the T2D+, T2D− and healthy groups were separated from each other in the OPLS-DA score scatter plots ($R2Y_{(CUM)}$ from 0.52 to 0.99, and $Q2_{(CUM)}$ from 0.20 to 0.64) (Supplementary Fig. S4). As shown in Fig. 2b and Supplementary Table S7, the PC (16:0/17:0), lysophosphatidylcholine (LPC) (18:2) and palmitoylcarnitine were higher in T2D group than in the healthy group (fold change = 4.51–13.84, Kruskal–Wallis

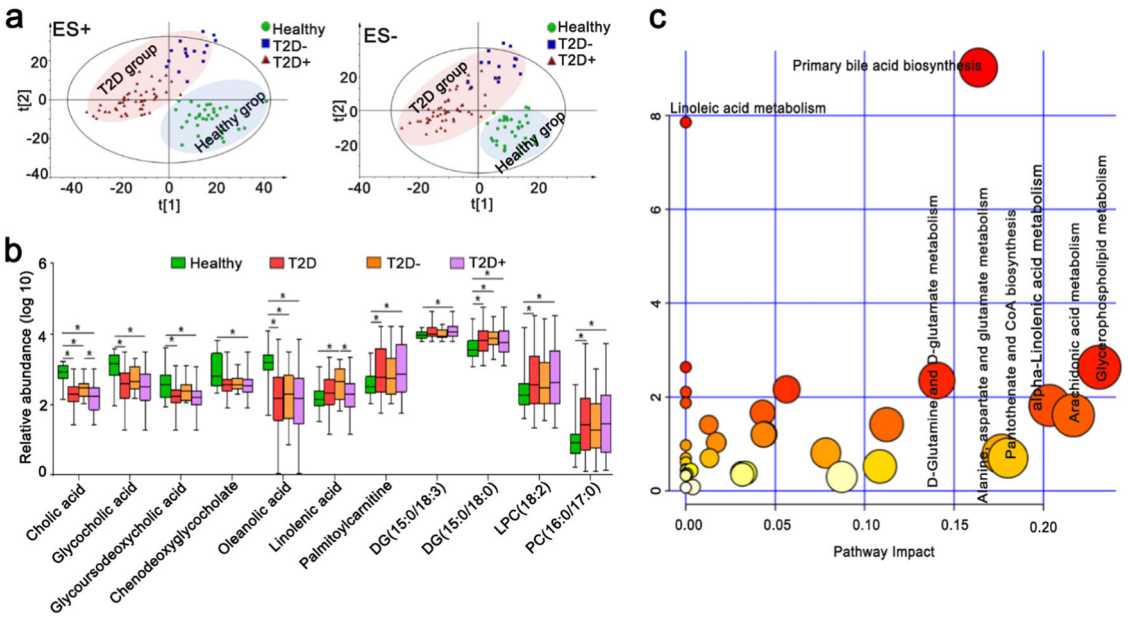

Fig. 2 Abnormal metabolic patterns of T2D and healthy groups. Partial least squares discriminant analysis (PLS-DA) score plots based on the metabolic profiles in positive (ES+) and negative (ES−) modes **a**. The box plot of differential faecal metabolites in T2D and healthy groups **b**. Kruskal–Wallis *H*-test, Bonferroni adjusted $^{*}P < 0.05$. LPC lysophosphatidylcholine, PC phosphatidylcholine, PE phosphatidylethanolamine, DG diacylglycerol. The disordered metabolic pathways are enriched based on differential metabolites between T2D and healthy groups **c**. Healthy healthy controls ($n = 35$), T2D− T2D patients without complications ($n = 16$), T2D+ T2D patients with complications ($n = 49$), T2D T2D− and T2D+ patients ($n = 65$)

H-test, $P < 0.05$; BMI and age adjusted $P > 0.05$), whereas some faecal bile acids, including cholic acid, glycocholic acid and glycoursodeoxycholic acid were significant lower in T2D group (fold change = −3.68 to −2.05, Kruskal–Wallis *H*-test, $P < 0.001$; BMI and age adjusted $P < 0.001$). When T2D+ group compared with T2D− group, the cholic acid was further decreased (fold change = −1.51, Kruskal–Wallis *H*-test, $P = 0.030$; BMI and age adjusted $P = 0.006$), which indicated that cholic acid might contribute to T2D complications. Sample size and power calculation using differential faecal metabolites as outcome variables showed that most of the power values were greater than 0.8 (Supplementary Table S8).

The differential metabolites among T2D patients and healthy subjects were related to bile acid metabolism, glycolipid metabolism and carbohydrate metabolism after pathway enrichment analysis (Fig. 2c). To be emphasised, these metabolic pathways were almost as same as the gut microbiota-gene functions, especially the carbohydrate metabolism and glycerophospholipid metabolism.

The gut microbiota and metabolic traits correlations of T2D patients and healthy subjects

From our available evidence, the gut microbiota and faecal metabolites were significantly altered between T2D patients and healthy subjects. Meanwhile, the SCFAs and some SCFA-producing bacteria were also remarkably changed among of them. Therefore, we performed WGCNA (see "Materials and Methods") to detect the comprehensive correlations between gut microbiota and faecal metabolites/clinical characteristics. We identified 44 different microbial modules (MMs). The complete list of taxa and their module organisation are shown in Supplementary Table S9. There were some interesting associations between the MMs and clinical characteristics, faecal metabolites and SCFAs (Fig. 3 and Supplementary Fig. S5). For example, the MM32, which contained family *Lachnospiraceae* genera *Lachnospiraceae NK4A136 group*, *Marvinbryantia* and *Blautia*, were negatively correlated with T2D groups and DBP, SBP, TC, TG and HDLC. The FBG and HbA_{1c} were positively correlated with the MM12, which contained genus *Akkermansia* in phylum *Verrucomicrobia*. Figure 3 shows that clinical characteristics, including BMI, WC, HC, WHR, DBP, SBP, TG, TC, HDLC and LDLC were positively correlated with MM35 and MM19, which contained genera *Prevotella* and *Prevotellaceae UCG-003* in *Bacteroidetes* and genera *Streptococcus*, *Weissella*, *Veillonella*, *Pseudobutyrivibrio* in *Firmicutes*. These two MMs were also positively correlated with faecal metabolites linolenic acid and LPC (18:2). The MM15 and MM16 contained families *Lachnospiraceae* and *Ruminococcaceae* were negatively correlated with acetate, while positively associated with LPC (18:2). Bile acids (cholic acid, glycoursodeoxycholic acid, chenodeoxyglycocholate and glycocholic acid) and SCFAs (acetate, propionate and butyrate) were negatively

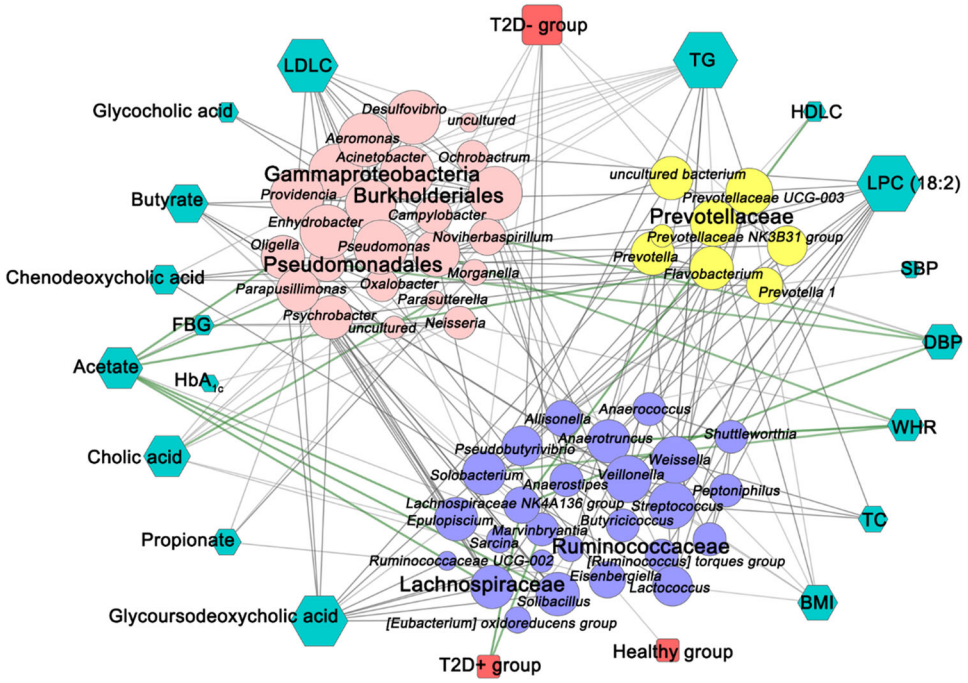

Fig. 3 The correlation network based on WGCNA between gut microbiota (circle) and metabolic traits (hexagon) in T2D and healthy groups (square). BMI body mass index, WHR waist-hip ratio, SBP systolic blood pressure, DBP diastolic blood pressure, FBG fasting blood glucose, HbA_{1c} glycated hemoglobin, TC total cholesterol, TG triacylglycerol, LDLC low density lipoprotein cholesterol, HDLC high density lipoprotein cholesterol, LPC lysophosphatidylcholine, Healthy healthy control, T2D− T2D patients without complications, T2D+ T2D patients with complications

correlated with MM38, MM14 and MM34, which contained microbiota in families *Lachnospiraceae*, *Ruminococcaceae*, *Planococcaceae* and *Prevotellaceae ect*.

From the results, some gut microbiota, such as genera in families *Lachnospiraceae* and *Ruminococcaceae* were not only significantly associated with SCFAs, but also correlated with clinical characteristics and several faecal metabolites, lipids and bile acids.

Discussion

In this study, we focused on the associations of gut microbiota with faecal metabolites and clinical characteristics using a cohort of 100 Chinese individuals. We sequenced the 16S rRNA V3-V4 region, analysed metabolites and SCFAs of faecal samples from 65 T2D patients without metformin or TCM treatment and 35 healthy subjects. We detected some meaningful associations between gut microbiota and metabolic traits. For example, the SCFA-producing bacteria were not only related to the SCFAs, but also to faecal bile acids and lipids. However, the gut microbiota's effect on the development of T2D and its complications remains controversial.

From our results, the concentrations of SCFAs, including acetate, propionate and butyrate were significantly decreased, while certain SCFA-producing bacteria in families *Ruminococcaceae* and *Lachnospiraceae* were significantly increased in T2D patients compared with healthy subjects, contradicting the findings of Qin et al. [32, 33]. The main reason for this inconsistency was the limitation of gene sequencing technology. The 16S rRNA sequencing could not acquire all the SCFA-producing bacteria. The second reason might be that Qin's research didn't consider treatment regimens, especially the metformin or TCM, which might affect the composition and function of gut microbiota in T2D patients [4, 34]. In addition, the concentrations of faecal SCFAs reflect a balance status between colonic production and absorption of SCFAs [35], or even reflect SCFAs absorption rather than its production [36]. It was concluded that SCFAs do not necessarily have the same metabolic influence as SCFA-producing bacteria on T2D [37]. More studies are needed to determine whether the kinetics of SCFA production and metabolism differ in T2D patients and healthy subjects.

In addition, we confirmed and supplemented previous correlations between certain SCFA-producing bacteria [38] and metabolic disorders in humans. For example, the abundance of family *Prevotellaceae* was significantly decreased in T2D patients with coronary artery disease and was positively correlated with BMI [39]. The *Prevotella species* was increased in obesity and hypertension subjects [40, 41]. The genera *Anaerostipes*, *Pseudobutyrivibrio*, *Streptococcus, Butyricicoccus* and *Veillonella* from families *Lachnospiraceae*, *Ruminococcaceae* and *Veillonellaceae* were increased in faecal samples of insulin resistance, T2D and cardiovascular disease patients, while these were positively correlated with FBG, HbA_{1c} and blood pressure [38, 39, 42, 43]. Most of the above bacteria are SCFA-producing bacteria [38], which were comprehensively correlated with T2D clinical characteristics, such as BMI, DBP, SBP, TG, TC, HDLC, LDLC and faecal LPC/PC

metabolites in our study. A previous study indicated that gut microbiota played a role in generation of TMAO from the PC and revealed the following metabolic pathway: PC → choline → TMA → TMAO [44]. TMAO is a cardiovascular and mortality risk of T2D complications independent of traditional hyperglycaemia, and TMAO is closely related to disordered gut microbiota [45]. Remarkably, LPCs were associated with cardiovascular risk and strongly age-dependent [46]. This might be one of the reasons for the changes in the statistical difference of faecal metabolites after adjusting for age and BMI, such as LPC (18:2) and PC (16:0/17:0). And the situation was also observed on certain microbiota in the phylum *Firmicutes*. Previous study reported that the phylum *Firmicutes* was altered with BMI, but not *Bacteroidetes* [47]. Thus, we concluded that the above metabolites and microbiota were closely related with adiposity and age. Since in human studies such controls are not possible, it is significant to split apart the contributions of age and BMI (and other factors) to clarify the relationships between the gut microbiota and PC on T2D/T2D complication. And it is vital to personalised diabetes care for the older or overweight/obese individuals with T2D since they have a high risk for the development of diabetic complication.

Bile acid are produced in the liver from cholesterol and metabolised in the intestine by the gut microbiota. These bioconversions modulate the signalling properties of bile acids via the nuclear farnesoid X receptor (FXR) and the G protein-coupled membrane receptor 5 (TGR5), which regulate numerous metabolic pathways in the host. And these processes can be altered by targeting the interplay between bile acids and the gut microbiota [48]. Studies confirmed that FXR and TGR5 signaling controls glucose homoeostasis by regulating glucagon-like peptide-1 (GLP-1) synthesis in intestinal *L* cells [49, 50]. In the human intestinal, the bile acids and the microbiota reciprocally control their composition. Indeed, bile acids act as antimicrobial agents by damaging bacterial membranes [51]. Gut microbial bile salt hydrolase (BSH) enzymes hydrolyse the conjugated bile acids to free bile acids, which then are further modified by microbiota to secondary bile acids during the feedback mechanism [52]. In our findings, some significantly decreased faecal bile acids in T2D patients were negatively correlated with some SCFA-producing bacteria, including families *Lachnospiraceae*, *Ruminococcaceae* and genera in phylum *Proteobacteria*, which have activation effects on BSH [53, 54]. Collectively, we hypothesised that there was interplay between the SCFA-producing bacteria and bile acids, which might alter the processes of glucose and lipid metabolism in T2D patients.

Moreover, the major trend of *Firmicutes* and *Bacteroidetes* or the ratio of *Firmicutes*/*Bacteroidetes* in case-control studies seem to be unstable [55]. Our results showed that the ratio of *Firmicutes*/*Bacteroidetes* was higher in the T2D groups compared with the healthy group, and the genera *Bacteroides* and *Prevotella* were the major contributors to the significant reduction of the phylum *Bacteroidetes* in T2D patients. Although *Bacteroides* and *Prevotella* seemed to be beneficial to T2D [40], *Bacteroides species* was reported to be an opportunistic pathogen of T2D [32], while *Prevotella* could induce insulin resistance [9] and was significantly increased in T2D with hypertension [56].

However, our study existed several limitations. First, the healthy control group screened by using FBG and HbA_{1c} was not measured by OGTT to exclude prediabetes or diabetes. Second, we could not eliminate the impact of concomitant therapies and food intake [57] on the faecal microbiota and metabolites in this cohort. Third, due to the small sample size, the correlations between the gut microbiota and metabolic traits and the predicted functions of microbiota were not able to adjust for age and BMI. Furthermore, data on faecal consistency were not available for our participants and this might have an impact on faecal microbiota and metabolites. And the serum levels of bile acids and SCFAs were not measured in the study, which might have hid more possible mechanisms underlying the correlations between gut microbiota and T2D metabolic characteristics [58].

Nevertheless, our study comprehensively described the correlations between disordered gut microbiota and faecal metabolites, which might provide significant evidences for subsequent studies on causality of gut microbiota in T2D. Although there are some controversies about the interplay between gut microbiota and T2D, it is undeniable that gut microbiota affects the metabolism of T2D patients by their related metabolites. Large longitudinal and interventional studies are further required.

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Author contributions X.L., H.L. and L.Z. conceived and designed the study. Material preparation and data collection were performed by L. Z., H.L. and S.C., L.Z., Y.P. and Y.Z. analysed and interpreted the data. The first draft of the manuscript was written by L.Z. and authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in the study involving human participants were in accordance with the ethical standards of the Committee on the Ethics of the Second Hospital of Shandong University (reference number: KYLL-2015(KJ)P-0103) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants or their legal guardians included in the study.

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