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Alterations in the gut microbiota and the faecal metabolomes are potentially associated with gestational diabetes mellitus through inflammatory response

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ABSTRACT

Gestational diabetes mellitus (GDM) is a disease of glucose intolerance that first occurs during pregnancy. Accumulating evidence underlined a link between gut microbiota dysbiosis and GDM, and microbial metabolites represent a unique way to explore microbiota-host interactions. However, the associations between changes in the gut microbiota and microbial metabolites and immune homeostasis in the GDM pathogenesis remain largely unclear. In this prospective study, the characteristics of gut microbiota in both first trimester (T1) and second trimester (T2) were investigated in 46 GDM patients and 44 matched controls. We comprehensively profiled the microbial metabolites using non-targeted metabolomics and quantitatively targeted metabolomics, measurements of inflammatory cytokines and biomarkers of intestinal barrier function, and combined with correlation analysis in T2. Gut microbiota dybiosis was observed in GDM patients in both T1 and T2, and was characterised by the enrichment of multiple potentially harmful bacteria, such as UBA1819 and *Erysipelatoclostridium*. Besides, alterations in the microbiota were accompanied by a disturbance in tryptophan metabolism, mainly manifested as a shift towards the production of more kynurenine and less indole derivatives. Most importantly, correlation network analysis indicated that overgrowth of potential pathogens and tryptophan metabolism disorder were associated with inflammatory imbalance and disrupted epithelial barrier in GDM patients. These findings provide a greater understanding of the pathogenesis and new targets for microecological interventions by mediating tryptophan metabolism in GDM.

1. Introduction

Gestational diabetes mellitus (GDM) is characterised by glucose intolerance with the first onset during pregnancy and is accompanied by chronic low-grade inflammation. It is typically diagnosed based on oral glucose tolerance test (OGTT) results at gestational weeks 24–28[1]. GDM is a common complication of pregnancy with a global prevalence of 25.1%[2]. It results in adverse maternal and neonatal outcomes, including pre-eclampsia, premature rupture of membranes and preterm delivery[1,3]. The hyperglycaemic states are usually transient during pregnancy, and restore to normoglycaemia after delivery. However, the risk of post-GDM women developing type 2 diabetes

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mellitus (T2DM) within 5 years is 20%–50%[4]. Disturbances in insulin resistance and insulin secretion are responsible for the occurrence of GDM[5]. However, the underlying pathological mechanisms warrant elucidation.

The evidence suggested that the low-grade inflammation contributes to GDM pathogenesis[6]. It is well established that gut microorganisms interact with their host and are essential for physiological processes, including inflammatory responses and host metabolism[7]. Recent advances have indicated that perturbations of the gut microbiota are associated with GDM[8]. There are different microbial remodeling patterns between women with and without GDM[9]. Most notably, a recent study showed that gut microbiome plays a role in inflammation-induced GDM pathogenesis using faecal microbiota transplant (FMT) of first trimester (T1) samples from GDM patients[10], which support the feasibility of elucidating the underlying pathological mechanisms of GMD insight into gut microbiota. Additionally, microbiota-derived metabolites interact with the host and transmit intestinal signals to the entire system, thus affecting metabolic function and host health. It is the avenue by which microbiota-host interactions occur[11], while the cross-talk between changes in the microbiota and GDM-related metabolites is largely unknown[8]. Further, the intestines are not only the largest ecological niche for commensal microbes, but are also essential sites for maintaining homeostasis and immunity[12]. However, it is uncertain whether there is a link between the microbiota and inflammation through microbial metabolites, thereby participating in the regulation of glycaemic regulation.

In this prospective study, the gut microbiota in both the T1 and second trimester (T2) was analysed to screen for differential microbiota with essential roles in the occurrence and development of GDM. We then comprehensively profiled faecal metabolome using untargeted metabolomics combined with targeted metabolite validation to identify GDM-associated metabolites in T2. To gain further biological insights, we profiled inflammatory cytokines and biomarkers of intestinal permeability, then employed an integrated network analysis to infer the possible associations between the gut microbiota, microbial metabolites, the inflammatory response, and gestational glycaemic status.

2. Materials and methods

2.1 Recruiting participants and design

The operation protocol was approved by the Research Ethics Committee of the Wuxi Xishan People's Hospital (Approval Code: xs2020ky010) and was registered with the Chinese Clinical Trial Registry (No. ChiCTR2100052265). All participants who agreed to participate in the study provided written informed consent prior to their enrollment.

This nested case-control study was implemented in 2021, and the recruitment process is illustrated in Fig. S1. Pregnant women aged 18–40 years who planned to undergo prenatal examination (≤ 12 gestational weeks) at the Xishan People's Hospital were invited to participate in the project. Women were excluded if they: 1) had a chronic medical condition, such as hypertension, or type 2 diabetes (T2D); 2) had a gestational disease other than gestational diabetes during pregnancy, including hyperemesis gravidarum, pre-eclampsia or intrahepatic cholestasis of pregnancy; 3) used antibiotics, probiotics, or medication during their pregnancy. In addition, pregnant women with prior history of pregnancy, childbirth and puerperal complications, severe disorders of eating behavior, and body mass index (BMI) ≥ 30 were excluded to minimize the impact brought by the additional factors on the study[13]. Participants had the same omnivorous dietary pattern, lived within 30 km of the study site, and have lived locally for at least 5 years, which reduced the effects of lifestyle habits and diet on the outcomes[14].

GDM was diagnosed by an obstetrician using 75 g OGTT performed at gestational weeks 24–28 (blood glucose thresholds for GDM diagnosis: 5.1 mmol/L for fasting, 10.0 mmol/L for 1 h, and 8.5 mmol/L for 2 h)[15]. Participants were divided into the healthy controls (HC) group and the GDM group based on their diagnosis results in the T2. When identifying healthy individuals for the control group, the general characteristics of healthy individuals, including age, gestational weeks and BMI, should be matched to those of GDM patients according to the methods mentioned in previous published studies[9,16]. Besides, pregnant women developed GDM were classified as GDM1 and GDM2 in the T1 and T2, respectively; normoglycemic participants were considered HC1 and HC2 in the T1 and T2, respectively.

2.2 Sample size estimation

The prior power analysis was calculated with G*Power 3.1. The estimated effect size of gut microbiota was based on Bray-Curtis distances (β-diversity) obtained from published studies investigating the characteristics of gut microbiota between controls and GDM patients[8-9,17]. To detect a statistical power of 80%, at least 32 participants per group were needed to achieve a moderate effect size (0.63) and a significance of 5%. The sample size assessment for untargeted metabolomics was based on the R^2_Y index in orthogonal partial least squares discriminant analysis (OPLS-DA), crucial metric reflecting the explanatory power of the model for the dependent variable. To detect a power of 0.8, at least 14 participants per group were required to obtain an R^2_Y value = 0.5 (converted to Cohen's d)[18-19] at $\alpha = 0.05$.

For multidimensional detection projects, including targeted metabolomics, inflammatory cytokines, and intestinal barrier function, we adopted an empirical standard, setting a minimum sample size of 30 per group. This choice was grounded in widespread clinical research settings, where it is generally accepted that a sample size of 30 patients per group is sufficient to assess phenotypic heterogeneity at the molecular level[20-23].

2.3 Sample collection

Serum samples were collected at gestational weeks 8–12 and 24–28 from pregnant women by professional nurses after an overnight fast, then plated at – 80 ℃ prior to analysis. Fresh stool samples were collected at gestational weeks 8–12 and 24 –28 using sterile stool collection tubes after which faecal samples were immediately transported to the laboratory and placed at –80 ℃ until analysis.

2.4 Faecal microbiome analysis

The Illumia Miseq sequencing protocol was conducted according to previously reported procedures[24]. In brief, DNA was extracted from stool samples with a FastDNA spin kit (MP Biomedical, Irvine,

CA, USA). 16S rRNA gene (V3–V4 region) amplicon sequencing was conducted with 341F and 806R primers. PCR products were purified and then quantitatively measured with a Qubit dsDNA assay kit (Life Technologies, Invitrogen, Carlsbad, CA, USA). Obtained amplicons were mixed in equal amounts after which they were sequenced with paired end on a MiSeq PE300 platform (Illumina, San Diego, CA, USA)[24].

Raw data were analysed through the QIIME2 pipeline (open-source, http://qiime2.org) and bioinformatic analyses were performed. *α*-Diversity was measured with Chao1, Shannon, and Simpson indices to assess richness and evenness at the genus level. *β*-Diversity was described according to the Bray-Curtis distance at the genus level and tested by a permutational multivariate analysis of variance. The differences in microbial composition were determined *via* Wilcoxon rank-sum test and linear discriminant analysis (LDA) effect size (LEfSe) (LDA > 2), and visually displayed through a taxonomic cladogram tree.

2.5 Untargeted metabolomics

Metabolites in stool were extracted according to previously reported methods[25-26], with some modification. Aliquoted freeze-dried faecal samples (approximately 50 mg) were transferred to a tube containing zirconia beads after which pre-cooled extraction solvent (ultra-pure water:methanol:acetonitrile = 1:2:2, *V*/*V*) was added. The above mixture was homogenized and then centrifuged at 15 000 × *g* for 10 min. Then resulting supernatant was moved to another centrifuge tube and concentrated. Dried samples were resuspended in 50% (*V*/*V*) acetonitrile and then moved to detection bottles for analysis by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) (Thermo Fisher Scientific, MA, USA). Specifically, equal volumes of each sample were mixed to prepare the quality control (QC) sample.

Untargeted metabolome profiling was performed with an UItiMate 3000 UPLC system equipped with a high-resolution Q Exactive (QE) MS and an UPLC HSS T3 column (2.1 mm ×100 mm, 1.8 μm; Waters, Milford, MA, USA). The detailed UPLC-MS operating conditions are provided in the Methods section of the Supplementary Material.

Data were further analysed by Compound Discovery 3.3 (CD 3.3, Thermo Fisher Scientific) and MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/)[27]. Peak alignment, peak picking, peak identification, normalisation, and the retention time (RT) and *m*/*z* data analysis were performed for each peak by CD 3.3. The mzvault, mzCloud, Chemspider, and Human Metabolome Database (HMDB) databases were applied for metabolite identification. Compounds with matching scores ≥ 80 in the mzCloud database were retained. Most importantly, the drift of each metabolic characteristic signal over time was independently corrected based on the locally estimated scatter plot smoothing curve[28]. Data QC was conducted by Pearson's correlation analysis and principal component analysis (PCA) plots to verify the stability of the non-targeted metabolomic profile. OPLS-DA was applied to the indicated the differences in the metabolic profiles between women with and without GDM in T2 and a permutation test was carried out 200 times *via* the SIMCA software (v17.0.2). Candidate biomarkers that distinguished the 2 groups in T2 were identified based on a variable importance plot (VIP) score > 1.0, $P < 0.05$, and fold change (FC) ≥ 2 or ≤ 0.5[29]. Pathway enrichment analyses were conducted with Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database.

2.6 Tryptophan quantification by UPLC-MS

The tryptophan metabolites were extracted as detailed above for untargeted metabolomic profiling. Samples were determined by an UItiMate 3000 UPLC system coupled to a QE and a UPLC BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm; Waters, Milford, USA)[30]. The detailed HPLC-MS/MS operating conditions were indicated in Supplementary Methods. Raw data files were processed with Thermo Scientific Xcalibur software (Thermo Fisher Scientific, MA, USA) combined with manual inspection of the retention time. Besides, the internal standard was used to correct the data for signal drift with time and metabolites losses caused by processing.

2.7 Short-chain fatty acid quantification by gas chromatography-mass spectrometry (GC-MS)

Faecal short-chain fatty acids (SCFAs) were extracted and measured as detailed in a previous report[31]. Briefly, 30 mg of the freeze-dried samples was soaked in saturated sodium chloride and homogenised. The homogenised samples were acidified using 40 μL of H_2SO_4 (10%, *V*/*V*). The acidified samples were then extracted using diethyl ether and centrifuged. The resulting supernatants were determined *via* gas GC-MS coupled to a Rtx-Wax column (column length: 30 m, inner diameter: 25 μm). Data files were analysed using Thermo Scientific Xcalibur software combined with manual inspection of the RT.

2.8 Blood index evaluation

The plasma glucose concentration was determined by Hitachi automatic biochemical analyzer (Hitachi, Tokyo, Japan). Insulin concentration was analysed using an Automatic immunoanalyzer (ARCHI TECT System, Kallang Place, Singapore). Routine blood examinations were carried with XN Series automated haematology analyser (SYSMEX, Kobe, Japan).

2.9 Measurements of inflammatory cytokines and biomarkers of epithelial barrier in serum

Inflammatory cytokines (interleukin (IL)-10, IL-1β, and tumor necrosis factor (TNF)-α) and biomarkers of epithelial barrier, including lipopolysaccharide (LPS), zonula occludens-1 (ZO-1), zonulin and occludin were quantified by enzyme-linked immunosorbent assay kits (Nanjing Senbeijia Biological Technology Co., Ltd., Nanjing, China) based on the instructions.

2.10 Statistical analysis

Statistical analysis was performed by Prism version 8.0.2 (GraphPad, San Diego, CA, USA), SPSS version 22 (IBM, Armonk, NY, USA) and R 4.0.3 (https://www.r-project.org/). Before further analysis, Shapiro-Wilk's test was performed to test for a normal

distribution. Parametric and nonparametric data were statistically analysed with a Student's *t*-test and a Mann-Whitney *U*, respectively. Categorical data were statistically analysed using Fisher's exact test. Data were indicated by mean values ± standard deviation (SD) or the median ± interquartile range or percentage. $P < 0.05$ was considered to represent statistical significance. Random forest classifer (R package mlr3verse) was performed to predict the discrimination between GDM2 and HC2 groups. A clustering correlation heatmap and a correlation network were constructed with OmicStudio tools (https://www.omicstudio.cn/tool) based on Spearman's correlation analysis.

3. Results

3.1 Clinical characteristics of the participants

A flow diagram of subjects recruitment is illustrated in Fig. S1. A total of 46 participants developed GDM, whereas 181 were normoglycaemic at gestational weeks 24–28. Then 44 healthy controls who matched the age, BMI, and gestational weeks of GDM patients, were included in the subsequent data analysis (Table 1). The number of participants in each group exceeded the estimated sample size. Besides, there were no apparent differences in gravidity, parity, family history of T2D, smoking history or drinking history between participants with and without GDM.

Table 1
Characteristics of participants.

Characteristic	GDM ($n = 46$)	HC ($n = 44$)	*P*-value
Age (years)	29.04 ± 3.96	27.93 ± 3.14	0.14
BMI (kg/m^2)	23.10 ± 4.08	21.93 ± 3.32	0.14
Education (years)	13.91 ± 2.96	13.27 ± 3.37	0.34
Smoking (current/former)	2 (4.34)	3 (6.82)	0.67
Smoking (never)	44 (95.66)	41 (93.18)	
Drinking (current/former)	8 (17.39)	7 (15.91)	0.85
Drinking (never)	38 (82.61)	37 (84.09)	
Parity	0.50 ± 0.62	0.57 ± 0.54	0.58
Parity (0)	21 (45.65)	26 (59.09)	0.21
Parity (> 1)	25 (54.35)	18 (40.91)	
Family history of type 2 diabetes	5 (10.87)	2 (4.55)	0.43
OGTT (0 h) (mmol/L)	4.75 ± 0.54	4.15 ± 0.37	< 0.001
OGTT (1 h) (mmol/L)	9.54 ± 1.85	6.43 ± 1.24	< 0.001
OGTT (2 h) (mmol/L)	8.24 ± 1.34	5.69 ± 0.81	< 0.001
HOMA-IR	2.20 ($n = 42$)	1.43 ($n = 41$)	0.006 9

Note: Data are presented as median (interquartile interval) or mean ± SD or *n* (percentage (%)). *P* value based on Mann-Whitney *U*-test or Student's *t*-test as appropriate. **P*-value based on Fisher's exact test. GDM, gestational diabetes mellitus; HC, healthy controls; BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance.

3.2 Perturbed gut microbiota in patients with GDM

16S rRNA gene sequencing analysis were conducted to explore the alterations in the gut microbiota of GDM patients. No obvious difference in α-diversity was found between women with and without GDM in either T1 or T2, suggesting that glucose metabolic disorders may not exert a major effect on microbial richness or diversity (Fig. 1A). Notably, PCoA analysis indicated that the microbiota composition of GDM patients showed a significant separation from normal individuals not only in the T2, but also in the T1 (Fig. 1B). Thus, the microbiota structures and signatures were markedly different between GDM patients and healthy individuals from early pregnancy.

Microbial analysis at the phylum level displayed that the dominant bacteria were Firmicutes and Bacteroidetes in each group, while the abundances of Bacteroidetes among the 4 groups were different (Fig. 1C). At the family level, Peptostreptococcaceae and Burkholderiaceae were more prevalent in the control groups in both T1 and T2, while Enterobacteriaceae was markedly enriched in the GDM groups in both T1 and T2 (Fig. 1D). Besides, LEfSe analysis was performed to detect the key gut microbiota components that discriminated GDM patients from healthy individuals at the genus levels (Figs. 2A and B). Totally, 7 discriminative taxa, namely, *Tyzzerella*, *Enterococcus*, *Flavonifractor*, *Hungatella*, *Stenotrophomonas*, *Erysipelatoclostridium*, and UBA1819, were identified between GDM2 and HC2 groups, and their abundances were all significantly increased in the GDM2 group compared with those in HC2 group. In particular, *Erysipelatoclostridium* and UBA1819 are not only biomarkers in T2 but also in T1 (Fig. 2C). The above observation indicated that perturbed gut microbiota occurred before the onset of hyperglycaemic symptoms. Additionally, the correlation between microbial disturbance and glycaemic dysregulation was indicated by Spearman's correction analysis. The abundances of *Flavonifractor*, UBA1819, *Erysipelatoclostridium*, *Tyzzerella,* and *Hungatella* were strongly positively correlated with the OGTT (2 h) result, and the abundances of *Flavonifractor* and *Enterococcus* were positively associated with the OGTT (1 h) and OGTT (0 h) results, respectively, illustrating that these microbiota components may fulfill critical roles in GDM pathogenesis (Fig. 2D). Besides, temporal changes in the gut microbial profiles at genus levels from T1 to T2 were examined in the GDM and HC groups, respectively. Some bacteria, such as *Lachnoclostridium*, *Lachnospira* and Lachnospiraceae ND3007 group showed similar temporal changes in the GDM and HC groups, while several bacteria, especially *Bilophila,* were only enriched in the GDM group in T2 compared with T1 (Figs. 2E and F). These findings indicated that gut microbiota play a critical role in the occurrence and development of GDM.

3.3 Faecal metabolome perturbations in patients with GDM

To elucidate the functional alterations in the GDM-associated microbiome and their relationship with the host, we conducted untargeted metabolomic analyses of faecal samples from GDM2 and HC2 groups. A total of 5 378 (4 607 with annotated metabolites) and 2 887 (2 451 with annotated metabolites) metabolic ion features were generated in positive electrospray ionization (ESI^+) and negative electrospray ionization (ESI^-), respectively. There were 402 metabolites that had level 2 confirmed structures in the mzcloud databases, based on MS/MS spectral best-match scores ≥ 80. The QC samples were closely clustered in ESI^+ and ESI^- modes, as illustrated in Figs. S2A-D, which reflected the stability and reliability of data. OPLS-DA analysis illustrated that faecal metabolome profiles considerably distinguished the GDM2 group from the HC2 group (Fig. 3A).

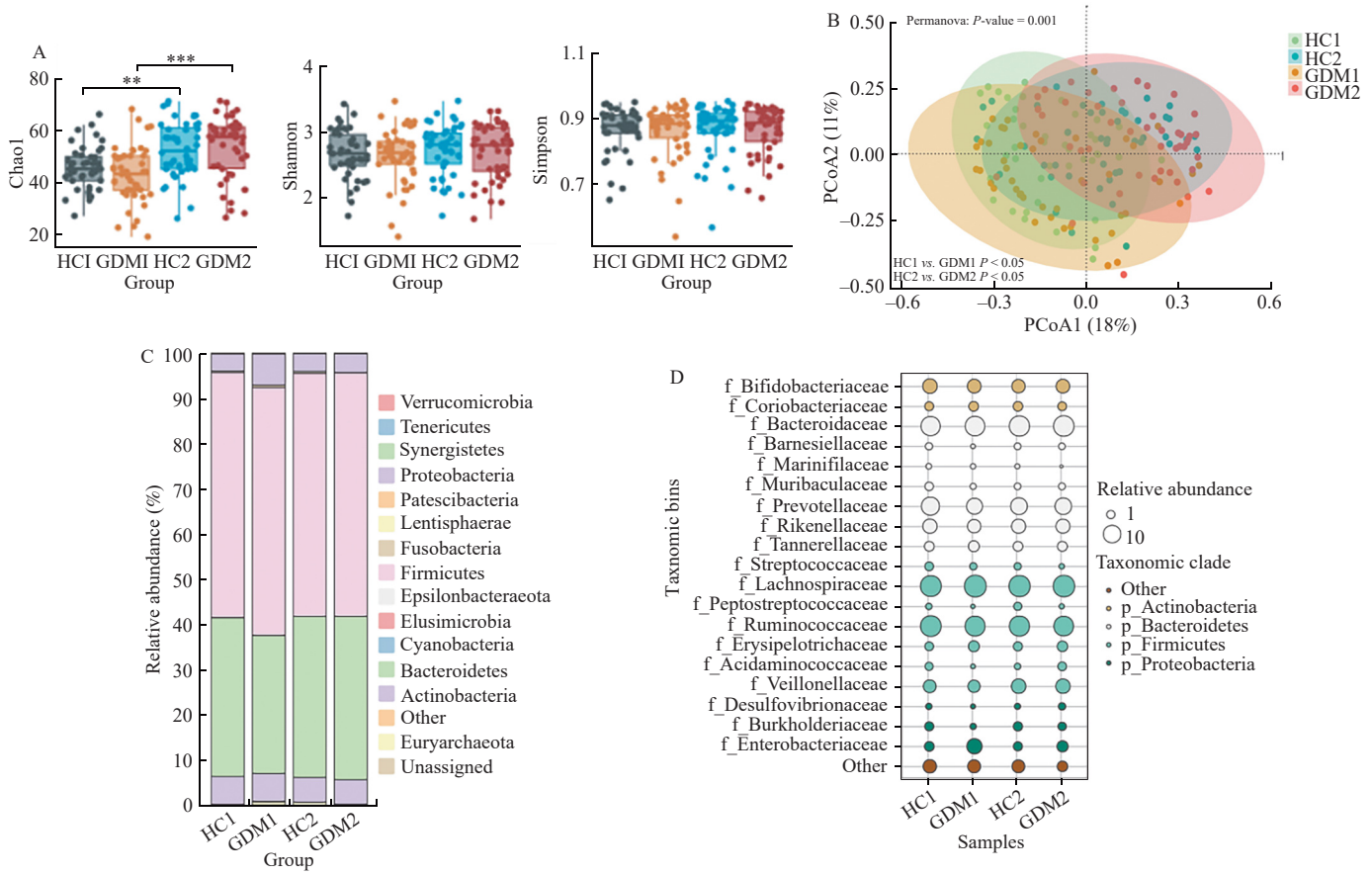

Fig. 1 The gut microbiota composition of women with and without GDM. (A) *α*-Diversity analysis indicated by Chao1, Shannon and Simpson indices. (B) *β*-Diversity characterized by a Bray-Curtis analysis. Relative abundance of microbes at (C) phylum and (D) family levels.

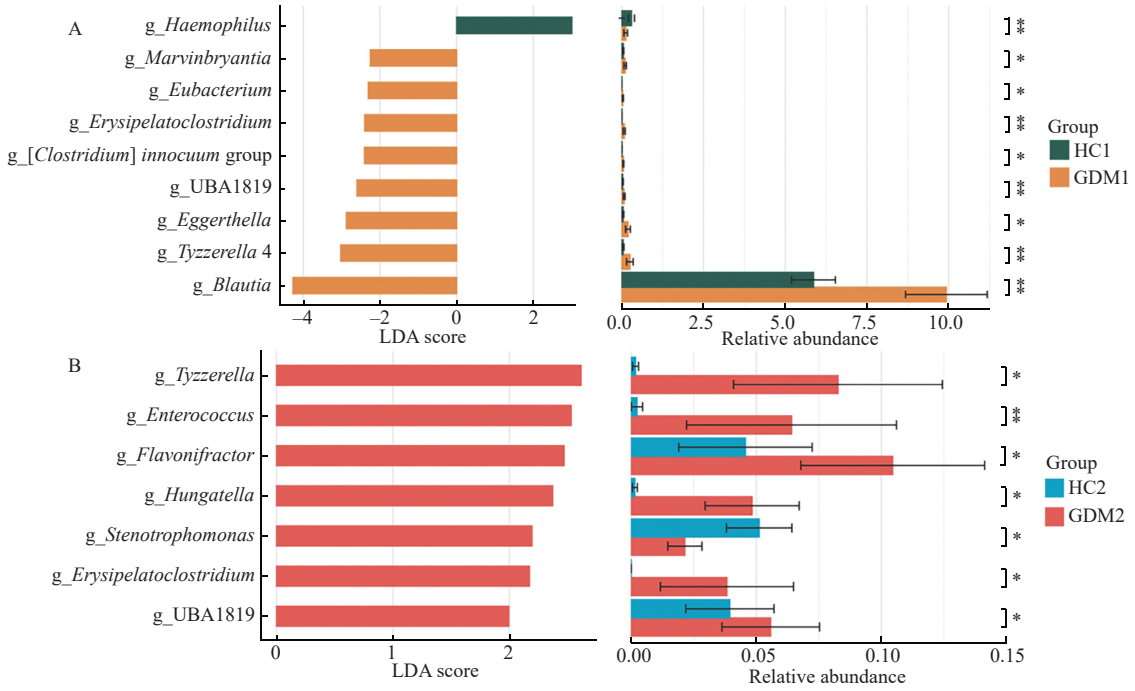

Fig. 2 Taxonomic biomarkers of women with and without GDM and correlation analysis of significant microbiota and glycemic traits. (A) Taxonomic biomarkers at genus level characterized by LEfSe between HC1 and GDM1 groups. The bar chart on the right represented the relative abundance of each taxon. (B) Taxonomic biomarkers at genus level characterized by LEfSe between HC2 and GDM2 groups. (C) The relative abundance of UBA1819 and *Erysipelatoclostridium*. (D) Spearman's correlation analysis of taxonomic biomarkers at genus level and glycemic traits in the T2. Taxonomic biomarkers at genus level characterized by LEfSe between T1 and T2 in the (E) HC group and (F) GDM group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The same below.

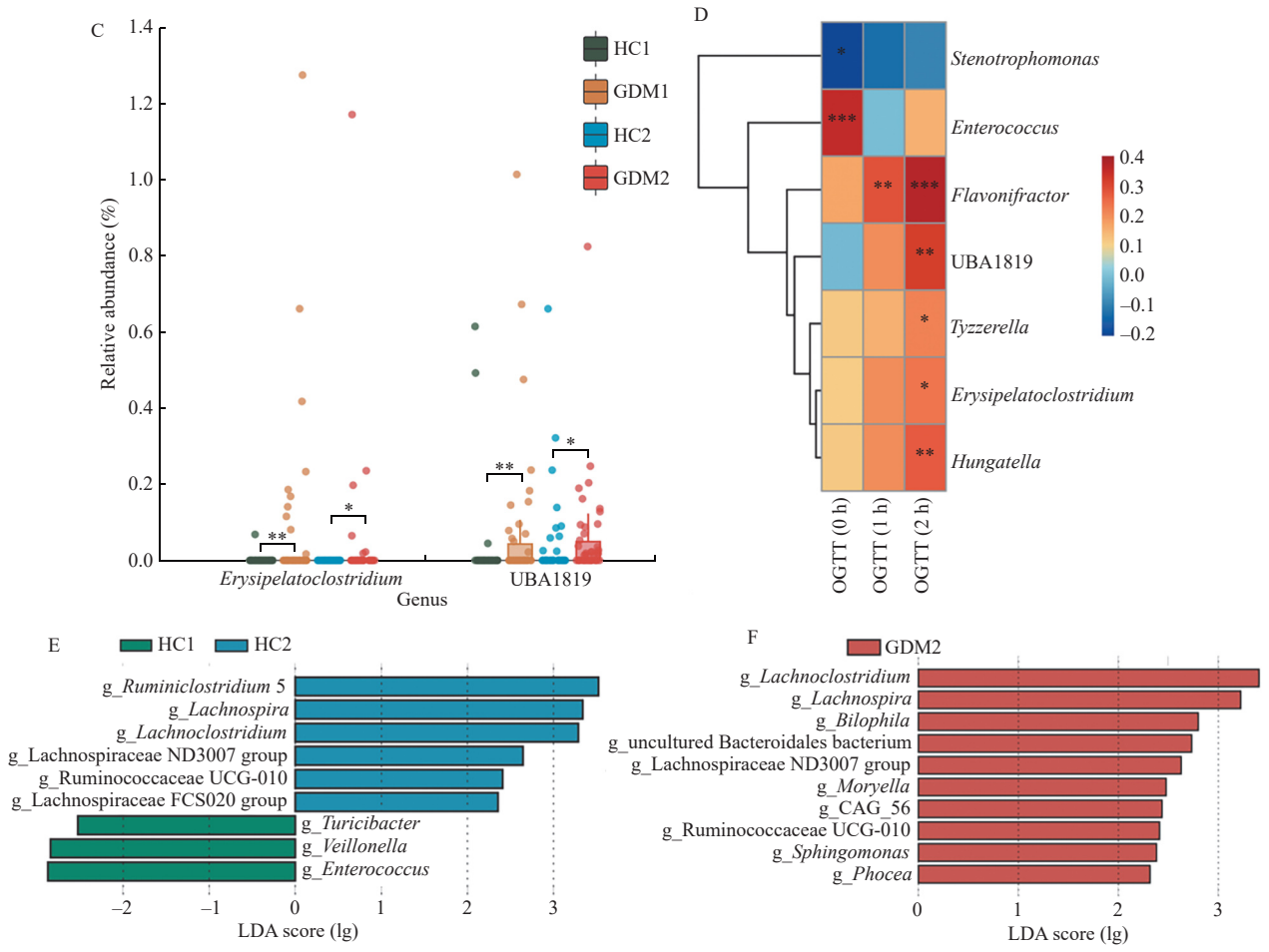

Fig. 2 (*Continued*)

The validity of the OPLS-DA model was verified through a random permutation test with 200 permutations. And the original points on the right were higher than all of the blue Q^2-values to the left in Fig. S2E, revealing that the model established in this study was reliable and not overfitting[32]. After adjusting for confounding factors, including the number of gestational weeks, age and BMI, there were 42 significantly differential metabolites between the 2 groups. These metabolites belonged to the categories of tryptophan, fatty acids, histidine, and steroid hormones (Fig. 3B). These data indicated that GDM status was associated with alterations in faecal metabolome profiles.

To further identify metabolites biomarkers contributing to the pathophysiological changes in GDM, an enhanced volcano plot combined model parameters of the multivariate analysis with the statistical parameters of the univariate analysis (FC and *P*-value) was employed. The results showed that 20 candidate biomarkers with significant changes (12 upregulated and 8 downregulated) were identified (Fig. 3C and Table S1). In particular, the concentrations of tryptophan and tryptophan metabolites were markedly different between GDM2 and HC2 groups. Functionally, these candidate biomarkers were mainly enriched in pathways of tryptophan metabolism, histidine metabolism, and aminoacyl-tRNA biosynthesis (Fig. 3D).

Untargeted metabolomics analysis is often applied to the initial discovery of differential metabolites. Targeted metabolomics is then required for the validation and accurate quantification of the biomarkers. There were apparent differences in the concentrations of tryptophan and its metabolites between participants with and without GDM in T2, and the microbial pathway with the largest enrichment ratio was tryptophan metabolism. Thus, targeted metabolomics was performed to quantify the faecal tryptophan metabolites and gain further insights into the pathogenesis of GDM. As illustrated in Fig. 4A, tryptophan and 12 tryptophan metabolites were measured. Of note, the concentrations of indole metabolites, especially indole-3-lactic acid (ILA), indole-3-acetamide (IA), and indole-3-propionic acid (IPA) were highly lower in patients with GDM than in healthy pregnant women, while the concentrations of 5-hydroxyindole acetic acid (5-HIAA) and kynurenine (Kyn) showed significant increases in women diagnosed with GDM. These data suggested that changes in the tryptophan metabolism may be implicated in the gestational glycaemic dysregulation.

In addition, we focus on the changes in the SCFAs, which are crucial metabolic products and have previously been reported to affect glucose homeostasis[33]. GC-MS analysis showed that the concentrations of acetate and butyrate were dramatically decreased in women with GDM relative to healthy controls in T2, while the concentrations of propionate, isobutyrate and isovalerate exhibited no changes (Fig. 4B). Thus, alterations in the concentrations of acetate and butyrate were associated with increased blood glucose concentrations.

Random forest analysis was performed[34], revealing that a combination of metabolic markers, including Kyn, 5-HIAA, ILA, IA, IPA, acetate, and butyrate, effectively distinguished GDM2 patients from healthy controls, with area under the curve (AUC) values 0.95 ($P < 0.001$)

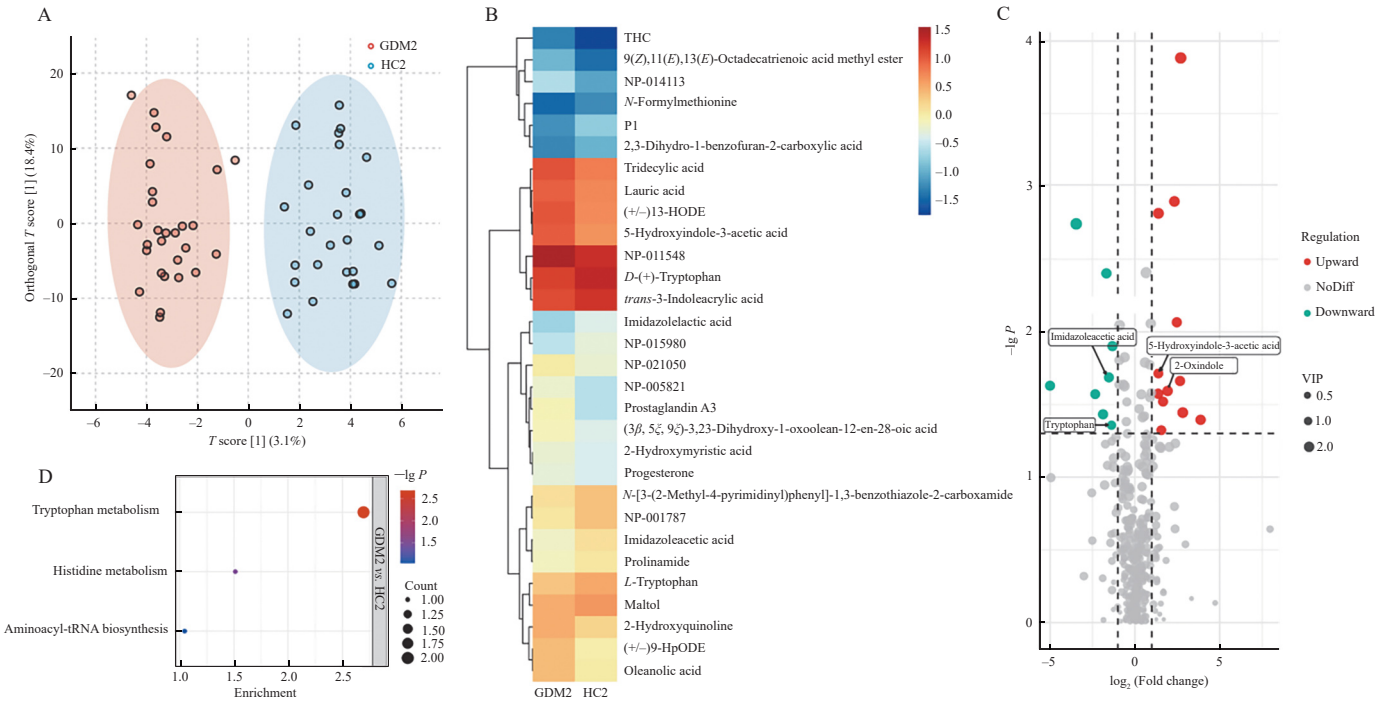

Fig. 3 Untargeted metabolome profiling of women with and without GDM. (A) OPLS-DA score plot. (B) Heatmap *via* hierarchical clustering with Ward method of top 30 changed metabolites. (C) Enhanced volcano plot. (D) Enrichment analysis of metabolic pathways based on KEGG database. P1, (2*S*,3*S*,4*S*)-3,4-dihydroxy-6-methoxy-3-methyl-7-methylidene-2-pentyl-2*H*,3*H*,4*H*,5*H*,6*H*,7*H*-pyrano[2,3-c]pyrrol-5-one. THC, tetrahydrocannabinol.

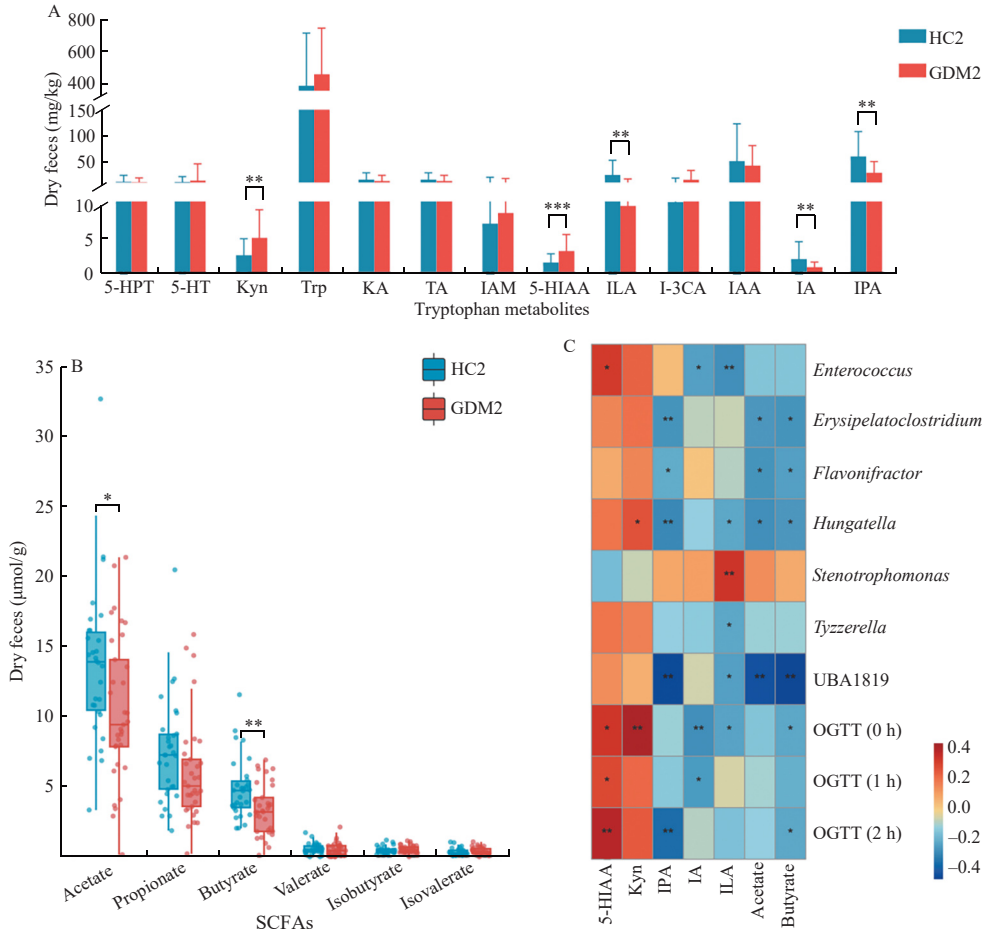

Fig. 4 Targeted metabolome profiling of tryptophan metabolites and SCFAs. (A) Quantification of tryptophan metabolites. (B) Quantification of SCFAs. (C) Spearman's correlation analysis integrated gut microbiota and glucose values with discriminative metabolites. **P* < 0.05; ***P* < 0.01.

after 10-fold crossvalidation (Fig. S3). These results suggested that discriminative metabolites could characterise the differences between GDM2 and HC2 groups.

Besides, we integrated gut microbiota and glucose values with metabolite levels to perform correlation analyses (Fig. 4C). IPA and butyrate levels were negatively correlated with the OGTT (2 h) result, while 5-HIAA concentration was positively linked to the OGTT (2 h) result. The IA level was inversely correlated with the OGTT (1 h) result, and IA, ILA, IPA and butyrate concentrations were negatively associated with the OGTT (0 h) result, but the Kyn concentration was positively correlated with the OGTT (0 h) result. Hence, strong positive correlations were observed between the levels of tryptophan metabolites and host glycaemic traits, indicating that tryptophan metabolites may involve in host glucose metabolism during pregnancy.

3.4 Participants with GDM exhibited increased intestinal permeability and inflammatory response

Notably, differentially abundant taxa associated with glycaemic status were predominantly Gram-negative pathogenic bacteria, such as *Hungatella* and *Stenotrophomonas*, and discriminative metabolites, including indole derivatives, were responsible for intestinal barrier function and the inflammatory response. Additionally, accumulating evidence indicated that increased intestinal permeability and low-grade inflammation contributed to glucose metabolic disorders[6]. We hypothesised that gut microbiota dysbiosis is implicated in glucose metabolic disorder by mediating inflammation and increased intestinal barrier. Thus, intestinal barrier related indicators (LPS, zonulin, ZO-1, and occludin), systemic inflammatory biomarkers (white blood cells (WBCs), neutrophils, lymphocytes, monocytes, and eosinophils), and inflammatory cytokines (IL-10, IL-1β, and TNF-α) were examined for participants in T2.

The numbers of the important serum inflammatory biomarkers, WBCs and lymphocytes were greatly enhanced in the GDM2 group when compared to those in the HC2 group (Fig. S4). Notably, the concentrations of TNF-α and IL-1β were remarkably higher in GDM patients than in control individuals ($P < 0.001$), while no significant alterations were observed in the levels of IL-10 (Figs. 5A-C). Serum levels of zonulin, LPS, and ZO-1, were remarkably elevated in the GDM2 group compared to the HC2 group, whereas there was no change in occludin concentration (Figs. 5D-G). Besides, a combination of inflammatory and intestinal barrier markers, including TNF-α, IL-1β, ZO-1, and zonulin, as determined by random forest analysis, can distinguish between GDM2 and HC2 groups with AUC values 0.88 ($P < 0.001$) after 10-fold crossvalidation (Fig. S5). These observations highlighted that low-grade inflammation and gut barrier dysfunction may contribute to the pathophysiological alterations in GDM.

3.5 Correlation network analysis of changes in the gut microbiota, microbial metabolites, and immune homeostasis with glycaemic traits

To further confirm the role of microbiota-metabolite-immune homeostasis in the pathogenesis of GDM, we systematically integrated glycaemic status and cytokines concentrations with gut microbiota and metabolite data based on a correlation network (Fig. 6). The abundances of microbes associated with gestational glycaemic dysregulation, such as, *Hungatella,* UBA1819 and *Flavonifractor*, were strongly correlated and may mutually promote the growth of one another. Moreover, *Hungatella* and ILA exhibited a high degree (the

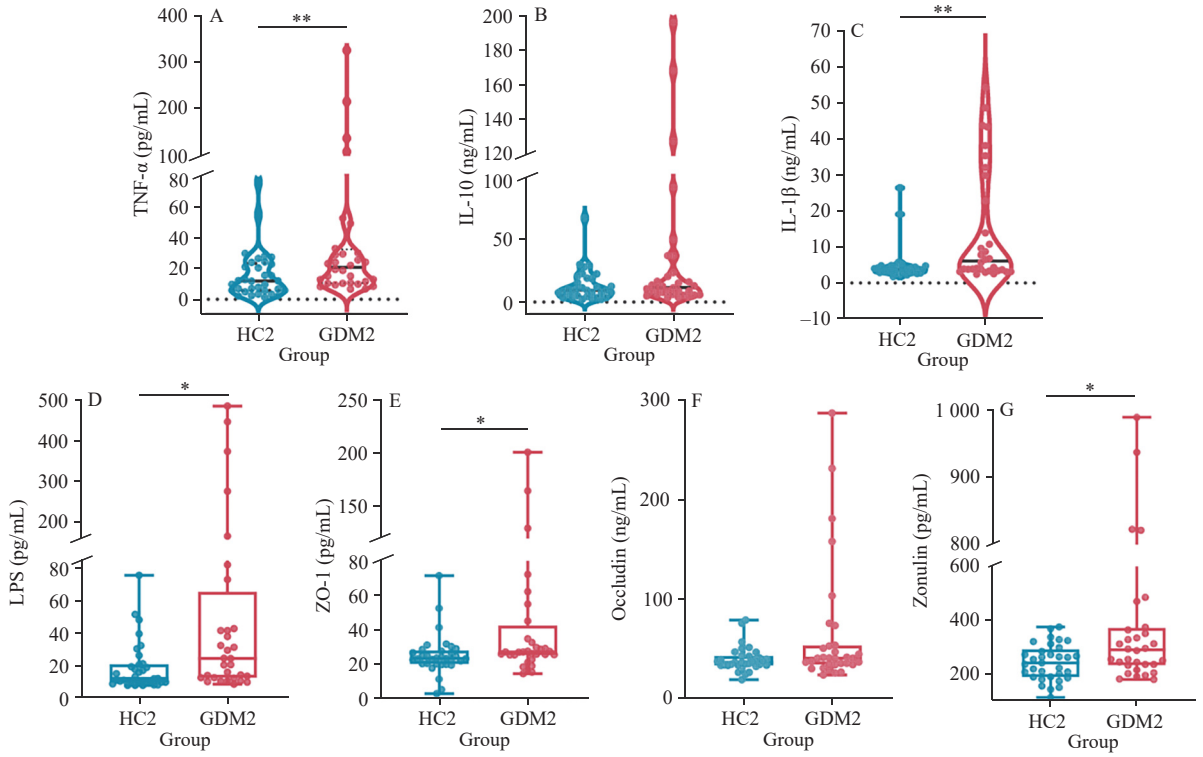

Fig. 5 Comparison of inflammatory cytokines and intestinal barrier biomarkers of women with and without GDM. (A) TNF-α. (B) IL-10. (C) IL-1β. (D) LPS. (E) ZO-1. (F) Occludin. (G) Zonulin.

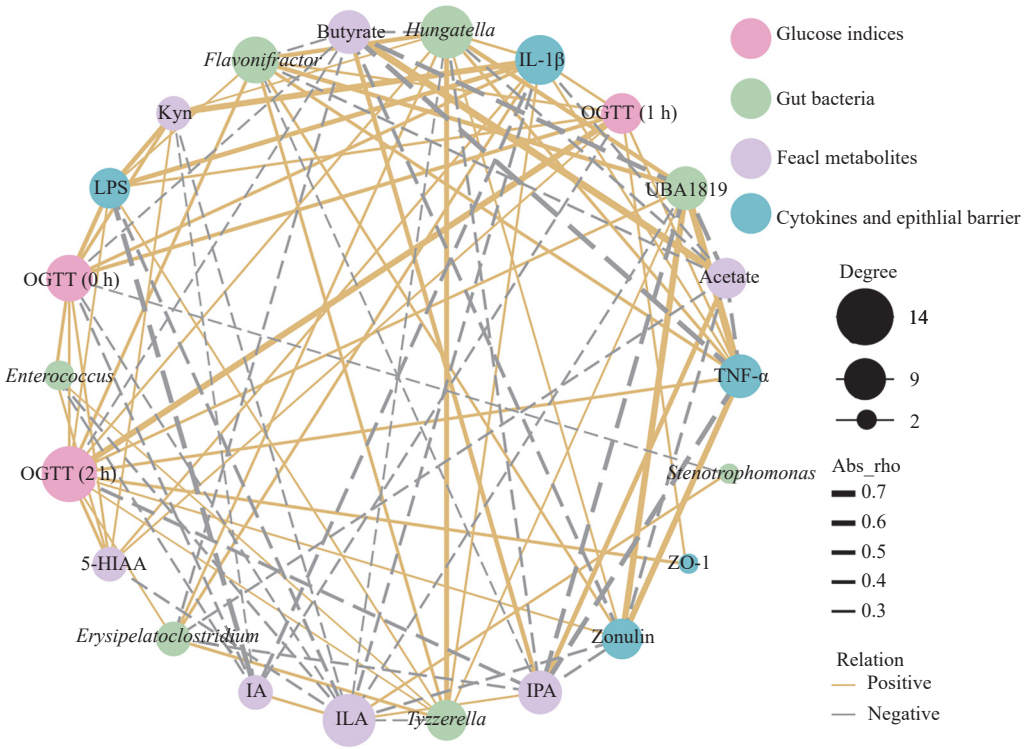

Fig. 6 Correlation network analysis of significant bacteria, metabolites, inflammatory cytokines, biomarkers of epithelial barrier and glucose indices. Only *P*-value < 0.05 displayed in the network.

number of connections it has to other nodes) in the network. Consistent with prior evidence[35], the concentrations of pro-inflammatory cytokines, including TNF-α and IL-1β, and intestinal barrier biomarkers (zonulin, ZO-1, and LPS) were inversely correlated with glycaemic status. Notably, the levels of *Hungatella* and Kyn were positively correlated with the IL-1β concentration, and the abundance of *Hungatella* was closely linked to the concentrations of Kyn and butyrate, which were also associated with abnormal glycaemic status. Moreover, the levels of UBA1819 and IPA were positively and negatively correlated with the zonulin concentration, respectively, and the abundance of UBA1819 was inversely linked to the IPA level, which both were also associated with the OGTT (2 h) result. Collectively, the correlation network analysis showed that alterations in immune homeostasis, microbial metabolites, gut microbiota, and glycaemic state in GDM were strongly correlated.

4. Discussion

GDM is an increasingly prevalent metabolic disease during pregnancy[2], while its pathogenesis remains unclear. Emerging evidences indicates that gut microbiome and its metabolism are potentially implicated in the process of host metabolic and immune homeostasis[7]. However, it is unclear whether there is a link between the microbiome and the faecal metabolome and inflammation with gestational glycaemic dysregulation. In this study, a combination of 'omics' techniques was used to identify the microbiome and metabolite signatures of the GDM2 and HC2 groups, which indicated their potential role in the GDM-associated inflammatory response and thus their contribution to GDM. The gut microbiome was also detected in T1 to screen for differential microbiota with critical roles in the occurrence and development of gestational glycaemic dysregulation.

Based on a nested case-control design, the gut microbiota profiles of participants with GDM and matched control individuals were determined in both T1 and T2. The composition of the gut microbiota showed apparent alterations before the diagnosis of GDM. At the family level, Enterococcaceae was markedly enriched in patients with GDM in both T1 and T2. Besides, *Erysipelatoclostridium* and UBA1819 were discriminative bacterial species in both T1 and T2. Apart from differential bacteria mentioned above, *Tyzzerella*, *Enterococcus*, *Flavonifractor, Hungtella,* and *Stenotrophomonas* were also the discriminative bacteria in T2. Moreover, there were positive associations between the abundances of these key differential bacteria (*Enterococcus*, *Flavonifractor*, *Tyzzerella*, *Hungatella*, *Erysipelatoclostridium*, and UBA1819) and gestational glycaemic status, as indicated by Spearman's correlation analysis. These findings were consistent with prior evidence that the elevated abundances of *Enterococcus* and *Flavonifractor* were related to impaired insulin resistance and aberrant glycaemic traits[36-37]. Importantly, *Flavonifractor* are commonly associated with metabolic disorders, including T2DM and obesity[38]. Steck et al.[39] showed that the *Enterococcus* may express metalloproteases to impair the gut barrier and transmit into the bloodstream in vulnerable hosts, thus inducing an inflammation reaction[40]. Additionally, *Hungatella* is a Gram-negative bacteria and enriched in numerous intestinal inflammatory diseases[41]. UBA1819, which has pathogenic potential, attributed to a series of inflammatory or immune diseases, including spinal cord injury[42] and type 1 diabetes with stratification by albuminuria[43]. Besides, *Erysipelatoclostridium* is a genus of opportunistic pathogens that are defined as typical inflammatory bacteria and are positively correlated with endotoxin production[44], which can activate the innate immune system and trigger an inflammatory response[45]. Miao et al.[46] suggested that the abundance of *Erysipelatoclostridium* was positively correlated with indicators of glucose homeostasis.

Specially, the relative abundance of *Bilophila*, which is a typical pro-inflammatory bacteria[47], increased dynamically as gestation progresses in the GDM group. It is postulated that the enrichment of multiple potential harmful bacteria and inflammatory-related bacteria in GDM patients exerted detrimental effects on gut health, causing inflammation and impairment of the gut barrier, which may eventually reduce insulin sensitivity indices and induce perturbations in gestational glucose metabolism.

To comprehensively characterize the changes in metabolic products and simultaneously determine how intestinal bacteria and metabolic products change in GDM, the untargeted metabolomics analysis combined with targeted metabolomics verification was performed. We found that tryptophan metabolism was the key metabolic pathway associated with GDM. Three major pathways of tryptophan metabolism contributing to serotonin, Kyn, and indole derivatives in the intestine are directly or indirectly controlled by the gut microbiome[48]. In this study, disordered tryptophan metabolism essentially converged to the decreased levels in indole derivatives, including ILA, IA, and IPA, and enhanced levels in 5-HIAA and Kyn in GDM patients compared to those in healthy controls. Consistent with the results of this study, recent studies have suggested that a shift in faecal tryptophan metabolism towards the production of more Kyn and less indole derivatives in patients with T2D compared with those in healthy controls[49-50]. Indole derivatives, such as IPA, IA, and ILA, can improve blood glucose levels and insulin sensitivity by promoting β-cell function, which is beneficial to the host metabolic health[51]. Besides, indole derivatives are agonists of pregnane X receptor, which serves as a regulator of mucosal integrity, and facilitates barrier intergrity *via* TLR4 signalling[52]. It should be highlighted that indole derivatives also act as ligands for the aromatic meridian receptor (AhR) to influence the Th17 cell/regulatory T cell balance and immune function, which is conducive to improvements in mucosal integrity and immune response[53]. Indole derivatives were directly transformed from tryptophan, and their production depends entirely on the gut microbiota[48]. Most importantly, correlation analysis indicated that the abundances of *Hungatella*, *Erysipelatoclostridium*, *Flavonifractor*, UBA1819, and *Enterococcus* were negatively associated with the concentrations of indole derivatives, including ILA, IA, and IPA. Evidence from the previous study supports the notion that *Erysipelatoclostridium* is related to tryptophan metabolism[54]. The enrichment of multiple opportunistic pathogens in GDM may trigger decreases in the levels of ILA, IA, and IPA by affecting the activity of enzymes associated with the conversion of tryptophan to indole metabolites[48,55], thus influencing the immune response and blood glucose levels during pregnancy. Patients diagnosed with a relapsing inflammatory disease are characterized by elevated Kyn level[56]. Moreover, the activation of tryptophan metabolism to Kyn is connected to chronic low-grade inflammation and probably interferes with insulin secretion signaling, thus contributing to an elevated risk of T2D[49]. Intestinal microorganisms can mediate indoleamine 2, 3-dioxygenase (IDO) activity to control the pathway by which tryptophan is metabolized into Kyn[48]. Notably, a significantly positive association between the abundance of *Hungatella* and Kyn level was identified by Spearman's correlation analysis. Gut microbiota dysbiosis may initiate IDO activity in GDM patients to increase Kyn level, thereby leading inflammatory responses and interference with gestational glucose metabolism.

Besides, butyrate was depleted in the GDM2 group, which has been reported to be related to the increased gut permeability and inflammation[57]. The butyrate level was negatively correlated with the abundance of *Hungatella*. Besides, it has been shown that *Hungatella* can influence butyrate synthesis[58]. We postulate that the disturbances in SCFAs and tryptophan metabolism elicited by microbiota perturbations appear to activate inflammatory responses and disrupt the gut barrier, thereby inducing insulin resistance and GDM. The serum inflammatory status and intestinal barrier function were evaluated to verify this hypothesis and obtain mechanistic insights into the role of the microbiota in GDM pathology. The levels of biomarker of systemic inflammation (WBCs and neutrophils), and pro-inflammatory cytokines (IL-1β and TNF-α) were significantly enhanced in the GDM2 group by comparison to HC2 group. It has been shown that serum LPS, ZO-1and zonulin levels can be used as biomarkers of intestinal barrier integrity[59-60]. We found that the serum levels of LPS, zonulin, and ZO-1 increased significantly in the GDM group. It confirmed that GDM is accompanied by increased intestinal permeability and inflammatory response[6]. And correlation network that integrated cytokines, gut microbiota and metabolites indicated that enriched multiple potential pathogens and changes in tryptophan-derived metabolites levels were associated with profiles of pro-inflammatory cytokines and gut barrier biomarkers in GDM patients.

Thus, a shift in faecal tryptophan metabolism towards more kynurenine production and less generation of indole derivatives induced by gut microbiota dysbiosis may lead to insulin resistance and gestational glycaemic dysregulation by triggering aberrant inflammatory responses and epithelial barrier impairment. Further, specifically manipulating intestinal tryptophan metabolism through microbiota-based approaches or providing indole-derivatives-enriched diets to mediate the inflammatory response and intestinal barrier function appear to be actionable therapeutic strategies for improving gestational glucose metabolism in GDM patients. Nevertheless, further research is warranted to determine the potential mechanism.

In the subsequent research, we will perform a shotgun metagenomic sequencing technology with high resolution to determine the specific species involved in the pathogenesis of GDM. Due to limitations in the remaining sample quantity, the sample sizes for all indices matches exactly that of the gut microbiota cannot be ensured. A study with a larger sample size and equal distribution of samples across all indices will facilitate obtaining more comprehensive information. It is necessary to expand the sample size to further investigate the role of gut microbiota in the etiology of GDM. Despite the strong biological plausibility of the findings reported herein, it is difficult to make causal inferences because of the observational nature of this study. However, the study showed the potential biological mechanistic hypotheses by incorporating multi-omics data, including microbiota, metabolism and host pathological phenotypes data. Techniques combining FMT with other relevant methods will be applied in the future to verify the findings.

5. Conclusion

Gut microbiota dysbiosis characterised by the enrichment of multiple potential pathogens has occurred prior to the onset of GDM, and patients with GDM have different metabolic signatures, mainly involved in tryptophan metabolism. Most notably, imbalanced tryptophan metabolism was found to be linked to gut microbiota dysbiosis, which may mediate immune homeostasis to affect gestational glycaemic regulation in GDM. Our findings provide a

valuable reference for understanding the pathogenesis of GDM from the perspective of the interaction between the microbiota and gestational glycaemic regulation. Additionally, mediating tryptophan metabolism to suppress inflammation may be a novel microecological intervention for GDM.

Declaration of interests

Gang Wang is an editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250232.

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