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Editorial Board Member of *World Journal of Diabetes*, Guo-Xun Chen, PhD, Associate Professor, Director, Department of Nutrition, The University of Tennessee, Knoxville, TN 37909, United States. gchen6@utk.edu

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ORIGINAL ARTICLE

Basic Study

Characterization of gut microbial and metabolite alterations in faeces of Goto Kakizaki rats using metagenomic and untargeted metabolomic approach

Jin-Dong Zhao, Min Sun, Yan Li, Chan-Juan Yu, Ruo-Dong Cheng, Si-Hai Wang, Xue Du, Zhao-Hui Fang

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Jin-Dong Zhao, Chan-Juan Yu, Ruo-Dong Cheng, Si-Hai Wang, Xue Du, Zhao-Hui Fang, Department of Endocrinology, The First Affiliated Hospital of Anhui University of Chinese Medicine, Hefei 230031, Anhui Province, China

Jin-Dong Zhao, Graduate School, Anhui University of Chinese Medicine, Hefei 230012, Anhui Province, China

Min Sun, School of Life Sciences, Anhui University, Hefei 230039, Anhui Province, China

Yan Li, Department of Infectious Disease, The First Affiliated Hospital of Anhui University of Chinese Medicine, Hefei 230031, Anhui Province, China

Corresponding author: Zhao-Hui Fang, PhD, Chief Doctor, Department of Endocrinology, The First Affiliated Hospital of Anhui University of Chinese Medicine, No. 117 Meishan Road, Hefei 230031, Anhui Province, China. fangzhaohui1111@163.com

Abstract

BACKGROUND

In recent years, the incidence of type 2 diabetes (T2DM) has shown a rapid growth trend. Goto Kakizaki (GK) rats are a valuable model for the study of T2DM and share common glucose metabolism features with human T2DM patients. A series of studies have indicated that T2DM is associated with the gut microbiota composition and gut metabolites. We aimed to systematically characterize the faecal gut microbes and metabolites of GK rats and analyse the relationship between glucose and insulin resistance.

AIM

To evaluate the gut microbial and metabolite alterations in GK rat faeces based on metagenomics and untargeted metabolomics.

METHODS

Ten GK rats (model group) and Wistar rats (control group) were observed for 10 wk, and various glucose-related indexes, mainly including weight, fasting blood glucose (FBG) and insulin levels, homeostasis model assessment of insulin resistance (HOMA-IR) and homeostasis model assessment of β cell (HOMA-β) were assessed. The faecal gut microbiota was sequenced by metagenomics, and

faecal metabolites were analysed by untargeted metabolomics. Multiple metabolic pathways were evaluated based on the differential metabolites identified, and the correlations between blood glucose and the gut microbiota and metabolites were analysed.

RESULTS

The model group displayed significant differences in weight, FBG and insulin levels, HOMA-IR and HOMA-β indexes ($P < 0.05$, $P < 0.01$) and a shift in the gut microbiota structure compared with the control group. The results demonstrated significantly decreased abundances of *Prevotella* sp. CAG:604 and *Lactobacillus murinus* ($P < 0.05$) and a significantly increased abundance of *Allobaculum stercoricanis* ($P < 0.01$) in the model group. A correlation analysis indicated that FBG and HOMA-IR were positively correlated with *Allobaculum stercoricanis* and negatively correlated with *Lactobacillus murinus*. An orthogonal partial least squares discriminant analysis suggested that the faecal metabolic profiles differed between the model and control groups. Fourteen potential metabolic biomarkers, including glycochenodeoxycholic acid, uric acid, 13(S)-hydroxyoctadecadienoic acid (HODE), N-acetylaspartate, β-sitostenone, sphinganine, 4-pyridoxic acid, and linoleic acid, were identified. Moreover, FBG and HOMA-IR were found to be positively correlated with glutathione, 13(S)-HODE, uric acid, 4-pyridoxic acid and allantoic acid and negatively correlated with 3-α, 7-α, chenodeoxycholic acid glycine conjugate and 26-trihydroxy-5-β-cholestane ($P < 0.05$, $P < 0.01$). *Allobaculum stercoricanis* was positively correlated with linoleic acid and sphinganine ($P < 0.01$), and 2-methyl-3-hydroxy-5-formylpyridine-4-carboxylate was negatively associated with *Prevotella* sp. CAG:604 ($P < 0.01$). The metabolic pathways showing the largest differences were arginine biosynthesis; primary bile acid biosynthesis; purine metabolism; linoleic acid metabolism; alanine, aspartate and glutamate metabolism; and nitrogen metabolism.

CONCLUSION

Metagenomics and untargeted metabolomics indicated that disordered compositions of gut microbes and metabolites may be common defects in GK rats.

Key Words: Type 2 diabetes mellitus; Gut microbial; Metabolites; Goto-Kakizaki rats; Metagenomics; Untargeted metabolomics

Core Tip: Studies have suggested that the gut microbial and metabolites play an essential role in Goto Kakizaki rats. The results revealed evidence of a decrease in *Prevotella* sp. CAG:604 and increases in *Lactobacillus_murinus* and *Allobaculum_stercoricanis*. Fourteen potential metabolism biomarkers included glycochenodeoxycholic acid, uric acid, N-acetylaspartate, β-sitostenone, sphinganine, 4-pyridoxic acid, 13(S)-hydroxyoctadecadienoic acid, and linoleic acid, *etc*.

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INTRODUCTION

In recent years, the incidence of type 2 diabetes (T2DM) has shown a rapid growth trend in China, where its current prevalence is 12.8%[1]. T2DM is a metabolic disorder whose major pathological hallmark is insulin resistance[2]. Goto-Kakizaki (GK) noninsulin-dependent rats are a valuable animal model for studies of T2DM[3]. At 4 wk of age, GK rats exhibit insulin resistance, basal hyperglycaemia development, increased hepatic glucose production, and impaired insulin secretion[4,5]. GK rats share common features with human T2DM. Therefore, the results obtained using GK rats as the research subjects will be more similar to those of from patients with T2DM than the results obtained using other systems and will thus provide more useful evidence for analysing the pathogenesis and treatment of T2DM.

The gut microbiota of the host constitutes a massive, complex microecosystem. Exploring the composition of the gut microbiota will improve our understanding of the relationship with the host as one of the key factors in host health or disease. Recent studies suggest that both humans and animal

models of T2DM and its complications, including GK rats, exhibit dysbiosis of the gut microbiota and further indicate that this characteristic microbiota imbalance, which may involve decreased bacterial diversity, contributes to the development of T2DM[6-8]. In addition, studies have shown that undesirable shifts in metagenomic- and microbiota-associated metabolite production may negatively impact glucose tolerance and insulin resistance[9].

Because the human dietary structure is complex and diverse, the changes in gut microbes and metabolites observed in different studies vary, even if the type and amount of the diets administered to patients with T2DM (*e.g.*, dietary fibre contents or low-carbohydrate diets) and nutritional advice are controlled[10-12]. In this study, we selected a fixed diet with balanced nutrient proportions for rats to avoid deviations in the results due to dietary differences. In animal models of diabetes, streptozotocin or alloxan is usually selected as an agent for damaging pancreatic function. This type of model is more similar to type 1 diabetes. In the classification of diabetes, T2DM accounts for approximately 90% of cases. Therefore, the further exploration of gut the microbes and metabolites in a T2DM model is desired. Moreover, a joint analysis of the gut microbiota and metabolite structure has not previously been conducted[13]. Therefore, the faeces of GK rats were collected as the study object, and this study aimed to systematically characterize the faecal gut microbes and metabolites of GK rats using metagenomic and untargeted metabolomic approaches and to analyse the relationship of gut microbes and metabolites with glucose and insulin resistance.

MATERIALS AND METHODS

Experimental design

Ten male GK rats (aged 5-6 wk) were procured from Changzhou Cavens Model Animal Co., Ltd. (Changzhou, China; certificate No. 202145537), and ten male Wistar rats (aged 5-6 wk) were purchased from Sipeifu (Beijing) Biotechnology Co., Ltd. (Beijing, China; certificate No. 110324210106676238). The rats were acclimated in a controlled laboratory (25 ± 2 °C temperature, 60% ± 5% humidity) with free access to breeding feed and water. The Animal Committee of Anhui University of Chinese Medicine (Hefei, China; Approval AHUCM-rats-2021133) approved the experiments.

The rats were fed growth and reproduction feed during the 1-wk acclimatization period and were observed for 10 wk. The fasting blood glucose (FBG) level was measured, and nine rats in the model group showed levels exceeding 11.1 mmol/L, whereas the FBG level of the other rat was not as high. Additionally, one rat in the control group escaped due to poor management, and the analysis was thus performed using nine rats from each group. The body weight and FBG were measured every two weeks. The FBG levels in blood sampled from the tail vein were measured with a glucose metre (ACCU-CHEK Performa, ROCHE, Basel, Switzerland) after food deprivation for 12 h overnight.

Sample collection

Eighteen rats were anaesthetized *via* the intraperitoneal administration of pentobarbital sodium (30 mg/kg, Merck, United States). Blood samples were collected in nonheparinized tubes and centrifuged to obtain serum. The indexes FBG, insulin, homeostasis model assessment of insulin resistance (HOMA-IR) and homeostasis model assessment of β cell (HOMA-β) were assessed. The faecal were placed in Eppendorf tubes, and the serum and faecal were stored at -80 °C[14].

Faecal metagenomic analysis

Total DNA was extracted from 1 g of faeces using a kit (Omega Bio-Tek, Norcross, GA, United States), and the concentration, purity and quality of the DNA was determined[15].

The extracted DNA (average length of 400 bp) was fragmented using a Covaris M220 ultrasonicator (Gene Company Limited, China). A paired-end library was constructed using NEXTFLEX Rapid DNA-Seq (Bioo Scientific, Austin, TX, United States). Sequencing was performed with an Illumina NovaSeq system (Illumina Inc., San Diego, CA, United States) using NovaSeq Reagent Kits according to the manufacturer's instructions[16]. The paired-end Illumina reads were trimmed of adaptors, and reads with low quality (length < 50 bp, quality value < 20, containing N bases) were removed by fastp[17].

Metagenomic data were collected using MEGAHIT. Contigs with a length ≥ 300 bp were selected as the final assembly output and used for further gene annotation. Amino acid sequences from the predicted open reading frames with a length ≥ 100 bp were retrieved and translated from the NCBI database[18]. The nonredundant gene catalogue was constructed using CD-HIT and aligned to high-quality reads using SOAP aligner.

Faecal metabolomic analysis

The supernatant was extracted from 200 mg of faeces and transferred to sample vials[19]. Two microlitres of a sample was separated with an HSS T3 column and used for LC-MS/MS analysis. Mass spectrometric data were collected using a UHPLC-Q Exactive system (Thermo Fisher Scientific, Waltham, MA, United States) with an electrospray ionization source operating in the positive- and

negative-ion modes. Data acquisition was performed in the data-dependent acquisition mode.

The raw LC-MS/MS data were preprocessed using Progenesis QI (Waters Corporation, Milford, MA, United States) software. Internal standard peaks and false-positive peaks were removed from the data matrix, redundant signals were removed, and the peaks were pooled. In addition, the metabolites were searched and identified in the HMDB, KEGG and Metlin databases.

Metabolites detected in at least 80% of any set of samples were retained[20]. After filtering, the metabolite response intensity of the mass spectrum peaks was normalized using the sum-normalization method. Moreover, variables with a relative standard deviation > 30% relative to the quality control samples were removed, and log10 logarithmization was performed to obtain the final data matrix for subsequent analysis.

Statistical analysis

Statistical and graphical analyses of the data were performed by Student's t tests and fold difference analysis using SPSS 23.0 (International Business Machines Corporation, NY, United States) and GraphPad Prism 9.0 (San Diego, CA, United States) software. The heatmap data were normalized *via* the z score method [$z = (x-\mu)/\sigma$] and graphically visualized using the pheatmap package.

Variance analysis was performed with the matrix file after data preprocessing. The R package ropls (Version 1.6.2) was used to perform orthogonal partial least squares discriminant analysis (OPLS-DA). The stability of the model was assessed by 7-cycle interactive validation. The significantly different metabolites were selected based on the following criteria: variable importance in the projection score (> 1) of obtained by OPLS-DA and the P value < 0.05 obtained by Student's t test[21].

The differential metabolites were screened and mapped to their biochemical pathways through metabolic enrichment and pathway analysis based on the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/). These metabolites were classified according to their functions. Significantly enriched pathways were identified using MetaboAnalyst based on degree centrality and Fisher's exact test (https://www.metaboanalyst.ca/). The molecular weights of potential biomarkers were obtained from biochemical databases (https://www.genome.jp/kegg/compound/). The data were analysed using the Majorbio Cloud Platform (https://www.majorbio.com) and Personalbio Cloud Platform (https://www.genescloud.cn/).

RESULTS

Body weight

The initial body weights did not significantly differ between the groups (P > 0.05). In the final weeks of the experimental period, the model group had a significantly lower weight than the control group (P < 0.05; Figure 1A).

FBG and insulin resistance

The FBG levels showed significantly differences from weeks 0 to 11, and over this time period, the significant difference became increasingly pronounced (Figure 1B). The mean FBG of the model group exceeded 11.1 mmol/L at week 5 and was close to 16.7 mmol/L at week 11.

Starting from week 11, the fasting insulin level of the model group was significantly higher than that of the control group (Figure 1C). Additionally, the HOMA-IR index of the model group was markedly higher than that of the control group from week 11 onwards (Figure 1D). Moreover, the HOMA-β index of the model group was significantly lower than that of the control group (Figure 1E).

Community structure of the gut microbiota

In total, 4.15×10^{10} raw bases (bp) and 4.33×10^{8} raw reads were obtained. Subsequently, 1.59×10^{8} nonredundant genes were predicted from the reads after quality control and the removal of host sequences. In total, five domains, 14 kingdoms, 181 phylum, 316 classes, 551 orders, 940 families, 2764 genus and 12302 species were identified. At the domain level, the gut microbiota comprised bacteria, archaea, eukaryotes, viruses and unclassified bacteria, and bacteria accounted for more than 99.67% ± 0.05% of the organisms in both groups.

A principal coordinate analysis of the distance matrix showed that the first principal component (PC1) and the second principal components accounted for 13.87% and 10.59% of the observed variation, respectively (Figure 2A). The boxplots illustrate the distribution of different groups on the PC1 axis and reveal that the greatest difference existed between the control and model groups (Figure 2B).

At the phylum level, Firmicutes, Bacteroidetes, Actinobacteria, Spirochaetes, Proteobacteria and Candidatus Saccharibacteria were identified as the main phylum (Figure 3A). The relative abundances of Firmicutes and Bacteroidetes were 54.85% and 27.16% in the control group and 57.75% and 23.82% in the model group, respectively. Although no significant differences were detected, a greater abundance of Firmicutes and a lower abundance of Bacteroidetes were found in the model group than in the control group.

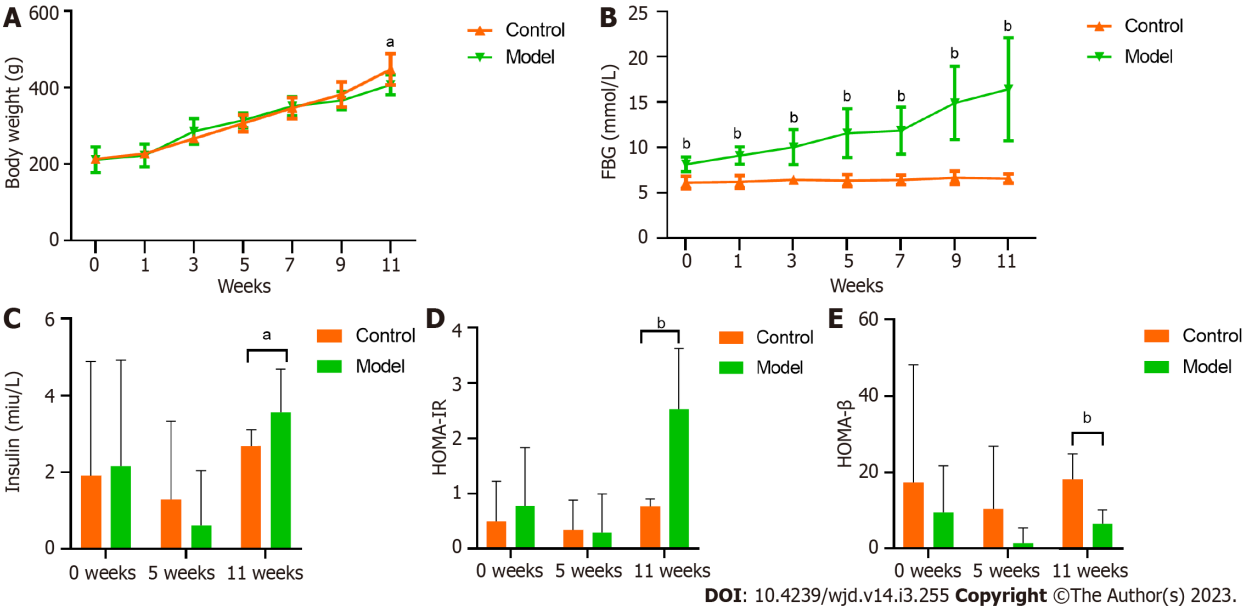

Figure 1 Comparison of body weight, fasting blood glucose and insulin resistance levels. A: Comparison of body weight; B: Comparison of fasting blood glucose levels; C: Comparison of insulin levels; D: Comparison of homeostasis model assessment of insulin resistance index; E: Comparison of homeostasis model assessment of β cell index. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control group. FBG: Fasting blood glucose.

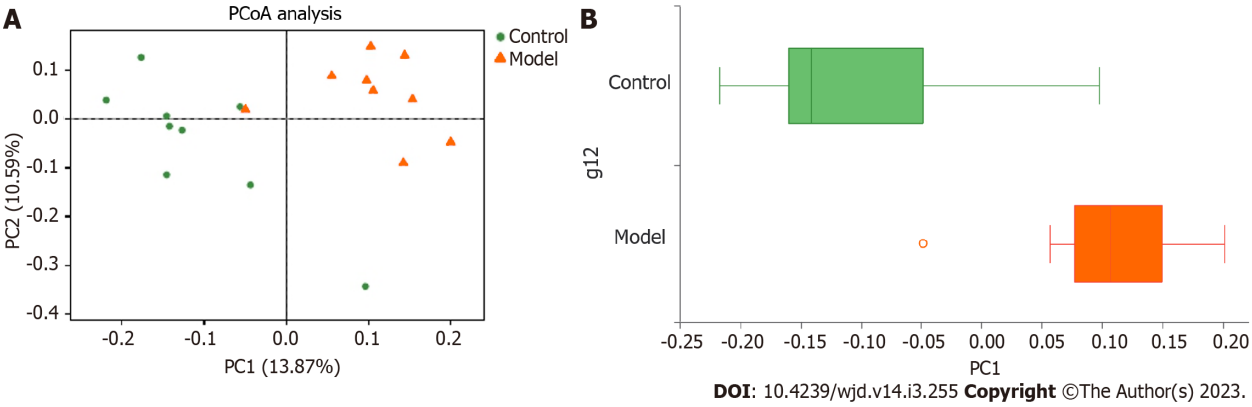

Figure 2 Comparison of the gut microbiota structure by principal coordinate analysis. A: Comparison of the gut microbiota structure by principal coordinate analysis; B: Distribution of different groups on the first principal component axis.

In addition to *Allobaculum*, which was the only genus showing a significant difference, *Prevotella*, *Bacteroides*, *Dorea*, *Phascolarctobacterium*, *Faecalibacterium* and *Ruminococcus* were enriched in the control group, and *Collinsella*, *Clostridium*, *Blautia* and *Lactobacillus* were the main components found in the model group (Figure 3B). Among the 30 species with the highest abundance, 3 species showed significant differences between the control and model groups ($P < 0.05$). A higher abundance of *Allobaculum stercoricanis* and lower abundances of *Prevotella* sp. CAG:604 and *Lactobacillus murinus* were found in the model group compared with the control group (Figure 3C).

The constructed heatmap revealed that FBG and HOMA-IR were moderately positively correlated with *Allobaculum stercoricanis* and moderately negatively correlated with *Lactobacillus murinus* ($P < 0.05$, $P < 0.01$; Figure 4A).

Metabolomic analysis

The faecal metabolic profile data of the control and model groups were separated by OPLS-DA model, which indicated that the positive- and negative-ion metabolic profiles of the samples differed between the two groups. The evaluation parameters of the OPLS-DA models from the positive- and negative-ion profiles showed values of $Q_2 = -0.199$ and $Q_2 = -0.068$, respectively, demonstrating good explanation and prediction with 200× permutation testing. For the positive-ion profiles, the component 1 showed a value of 14.00%, and the orthogonal component 1 showed a value of 30.70%. For the negative-ion profiles, the component 1 showed a value of 13.60%, and the orthogonal component 1 showed a value of 32.10% (Figure 5A-D).

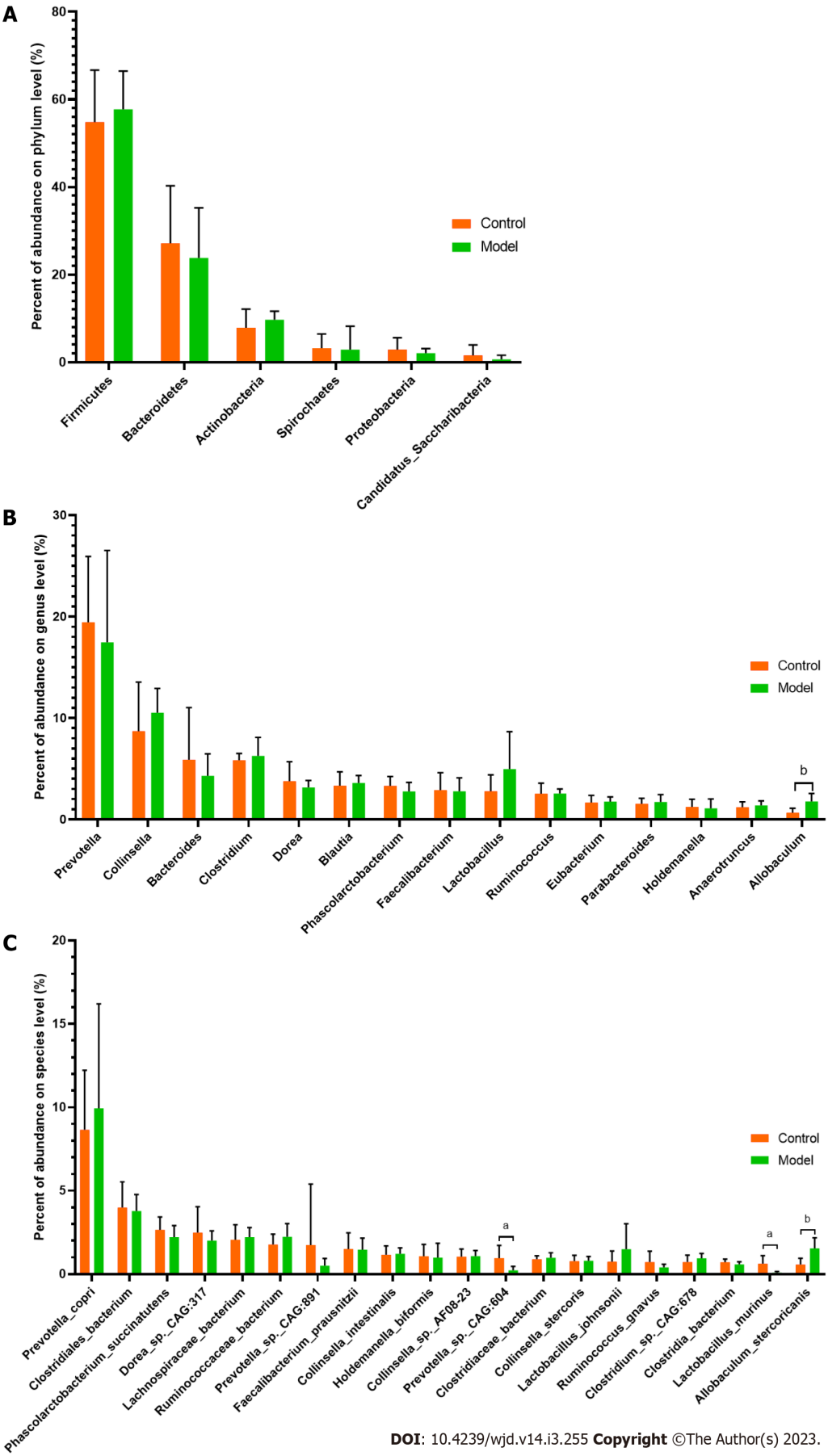

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Figure 3 Comparison of the relative abundances of phylum, genus and species. A: Comparison of the relative abundances of phylum; B: Comparison of the relative abundances of genus; C: Comparison of the relative abundances of species. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control group.

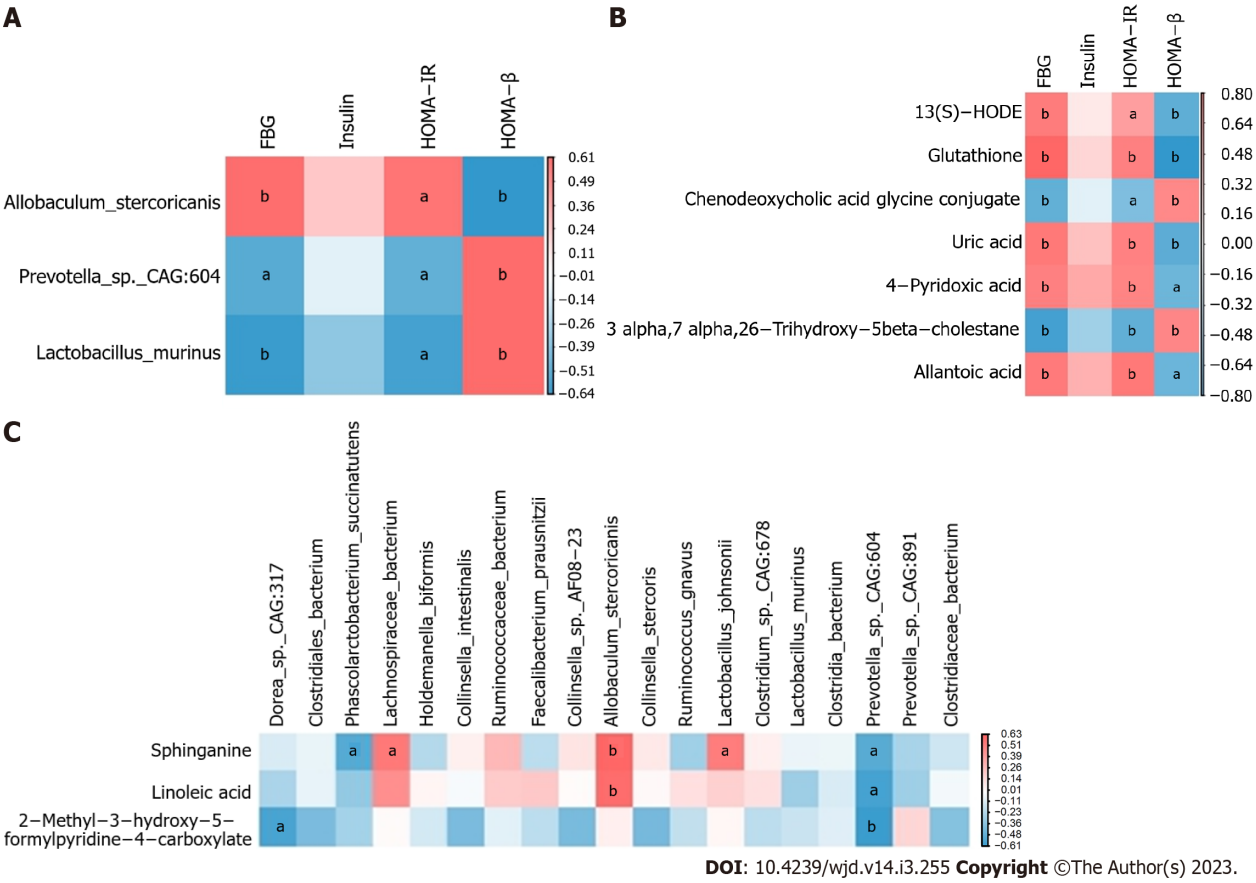

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Figure 4 Correlation the relationship between glucose factors, gut microbiota at the species level and metabolites. A: Correlation between glucose factors and the gut microbiota at the species level; B: Correlation between glucose factors and metabolites; C: Correlation between metabolites and the gut microbiota at the species level. [a]$P < 0.05$, [b]$P < 0.01$ *vs* the control group. The range of r values is noted. FBG: Fasting blood glucose.

We identified 815 positive-ion and 678 negative-ion metabolites, and 596 metabolites were found in the KEGG compound database. The differential metabolites (including 145 positive- and 106 negative-ion metabolites or 180 upregulated and 71 downregulated metabolites) were filtered in volcano plots (Figure 6A). The most reasonable molecular formula was obtained from a search of the KEGG Compound database. According to the abovementioned principle, 14 potential metabolic biomarkers were identified in GK rats (Table 1).

The heatmap revealed that FBG and HOMA-IR were positively correlated with 13(S)-hydroxyoctadecadienoic acid (HODE), glutathione, uric acid, 4-pyridoxic acid and allantoic acid and negatively associated with 3-α, 7-α, 26-trihydroxy-5-β-cholestane and chenodeoxycholic acid glycine conjugate ($P < 0.05$, $P < 0.01$; Figure 4B).

The heatmap also showed that sphinganine and linoleic acid were positively correlated with *Allobaculum stercoricanis* ($P < 0.01$) and that 2-methyl-3-hydroxy-5-formylpyridine-4-carboxylate was negatively associated with *Prevotella* sp. CAG:604 ($P < 0.01$; Figure 4C).

The KEGG compound IDs and compound names of endogenous metabolites were then introduced into the MetaboAnalyst 5.0 system for pathway and visual analyses. Based on the faecal biomarker data, the enriched pathways were found to include vitamin B6 metabolism; primary bile acid biosynthesis; arginine biosynthesis; purine metabolism; alanine, aspartate and glutamate metabolism; linoleic acid metabolism; and nitrogen metabolism. Among these pathways, six pathways were found to show differences (Figure 6B; Table 2). Figure 7 illustrates the key potential proteases of the metabolic pathways related to hyperglycaemia levels based on the KEGG database.

DISCUSSION

The aetiology and pathogenesis of T2DM are complex, and most experts believe that this disorder is a clinical syndrome caused by genetic and environmental factors. Because GK rats develop spontaneous diabetes and show insulin resistance, these rats constitute one of the best models for the study of T2DM. In clinical practice, we have found that some patients who suffer from T2DM do not consume a high-sugar and high-fat diet. Therefore, this study did not use a high-sugar and high-fat diet, which reduced

Table 1 Potential metabolism biomarkers identified

Metabolite	Class	KEGG compound ID	Mass M/Z	Mode	Formula	Retention time (min)	Change trend (model/control)
Glycochenodeoxycholic acid	Bile acids	C05466	414.2993	Pos	C26H43NO5	5.333	Down
Uric acid	Benzenoids	C00366	169.0355	Pos	C5H4N4O3	1.3882	Up
Glutathione	Amino acids	C00051	371.1005	Pos	C10H17N3O6S	1.0579	Up
Glycocholic acid	Bile acids	C01921	446.2898	Neg	C26H43NO6	5.3482	Down
B-sitostenone	Steroid	C00014	413.3772	Pos	C29H48O	9.9336	Up
Allantoic acid	Amino acids	C00499	175.0461	Neg	C4H8N4O4	0.6403	Up
N-acetylaspartate	Amino acids	C01042	174.0396	Neg	C6H9NO5	1.1621	Up
N-acetyl-l-glutamic acid	Amino acids	C00624	188.0553	Neg	C7H11NO5	1.4766	Up
Sphinganine	Amines	C00836	302.3045	Pos	C18H39NO2	6.9062	Up
4-pyridoxic acid	Pyridine carboxylic acids and derivatives	C00847	184.0604	Pos	C8H9NO4	1.6417	Up
2-methyl-3-hydroxy-5-formylpyridine-4-carboxylate	Pyridine carboxylic acids and derivatives	C06050	180.029	Neg	C8H7NO4	2.2041	Up
13(s)-HODE	Fatty acids	C14762	279.2311	Pos	C18H32O3	7.4255	Up
Linoleic acid	Linoleic acids and derivatives	C01595	281.2469	Pos	C18H32O2	8.1846	Up
3-α, 7-α, 26-trihydroxy-5-β-cholestane	Bile acids	C05444	419.3521	Neg	C27H48O3	8.5577	Down

Table 2 Metabolism pathway enrichment analysis

Pathway name	Match status	*P* value	-log (p)	Impact	Pathway level
Arginine biosynthesis	2/14	0.0055854	2.2529	0.19797	Amino acid metabolism
Primary bile acid biosynthesis	3/46	0.0056824	2.2455	0.05721	Lipid metabolism
Purine metabolism	3/65	0.014896	1.8269	0.0	Nucleotide metabolism
Alanine, aspartate and glutamate metabolism	2/28	0.021712	1.6633	0.09776	Amino acid metabolism
Linoleic acid metabolism	1/5	0.04129	1.3842	1.0	Lipid metabolism
Nitrogen metabolism	1/6	0.049357	1.3066	1.0	Energy metabolism
Vitamin B6 metabolism	1/9	0.073183	1.1356	0.0	Metabolism of cofactors and vitamins
Glutathione metabolism	1/28	0.21173	0.67421	0.25596	Metabolism of other amino acids
Glyoxylate and dicarboxylate metabolism	1/32	0.23835	0.62279	0.0	Carbohydrate metabolism
Glycine, serine and threonine metabolism	1/33	0.24487	0.61107	0.00245	Amino acid metabolism
Biosynthesis of unsaturated fatty acids	1/36	0.26413	0.57818	0.0	Lipid metabolism

the impacts of excess nutrition on glucose. Studies have shown that the glucose levels of 14- to 16-wk-old GK rats meet the diagnostic criteria for T2DM[3]. In this study, at 14 wk, the GK rats appeared to drink water and urinate more than the control rats, and their hair colour turned yellow. Before 12 wk, the GK rats were heavier than the control Wistar rats. However, at 14 wk, the body weight of the GK rats was lower than that of the control rats, and the FBG level of the former exceeded 11.1 mmol/L. By the 16th week, the body weight of the GK rats was further reduced, and the glucose level was further increased to levels exceeding 16 mmol/L. Furthermore, the increase in glucose was related an increase in the HOMA-IR index and a decrease in the HOMA-β index. This finding may be related to the observed decrease in the numbers of pancreatic β-cell islets, the irregular shape of the islets, and the presence of amyloid degeneration. Some researchers believe that weight loss is related to a reduction in the Firmicutes/Bacteroidetes ratio, but this phenomenon is not observed universally[22,23] and was not found in our research. Muñoz-Garach *et al*[24] believe that weight loss is related to a reduction in the level of bile acid, and the results of the present study are consistent with this view.

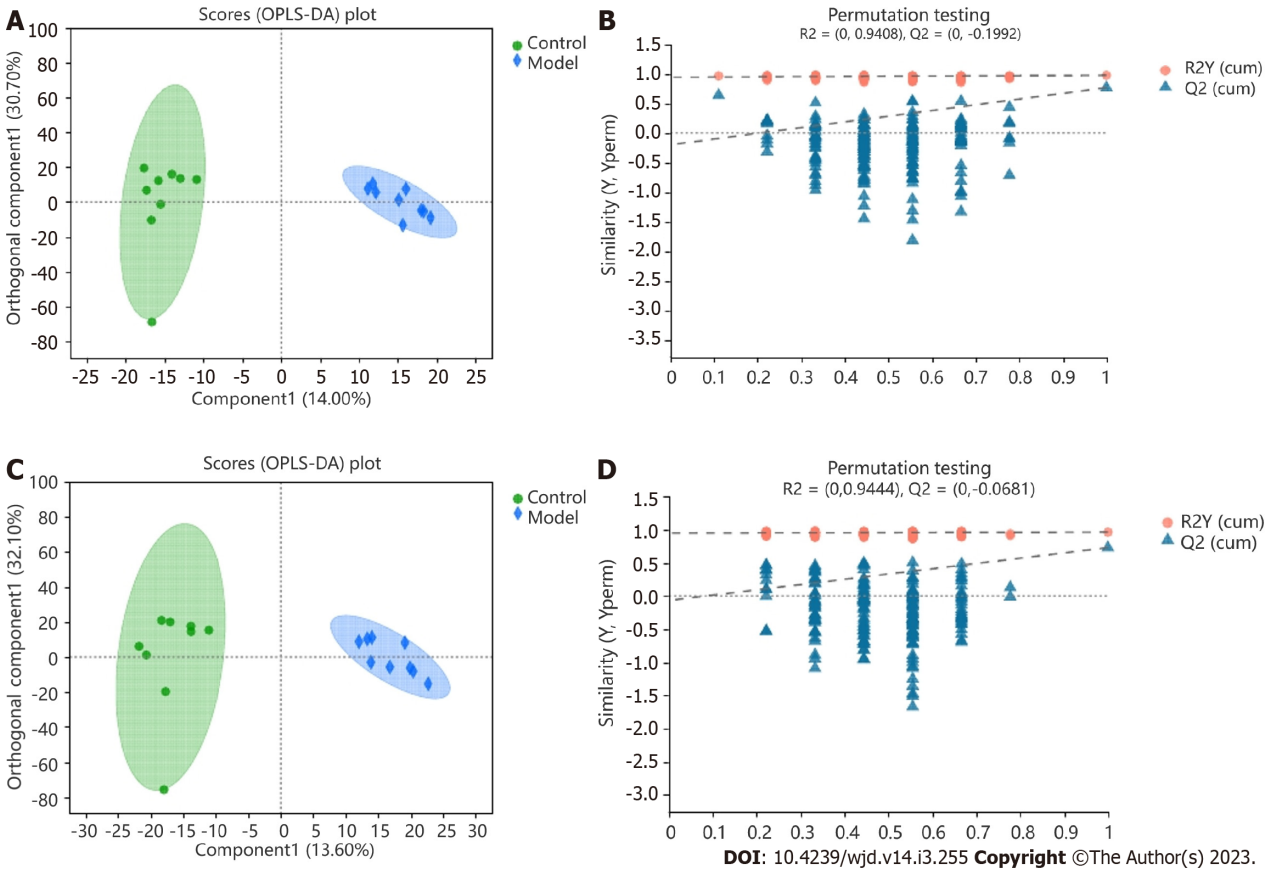

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Figure 5 Comparison of the metabolite profiles by orthogonal partial least squares discriminant analysis and model validation. A: Comparison of the positive-ion metabolite profiles by orthogonal partial least squares discriminant analysis (OPLS-DA); B: Positive-ion metabolite profiles with OPLS-DA model validation; C: Comparison of the negative-ion metabolite profiles by OPLS-DA; D: Negative-ion metabolite profiles with OPLS-DA model validation.

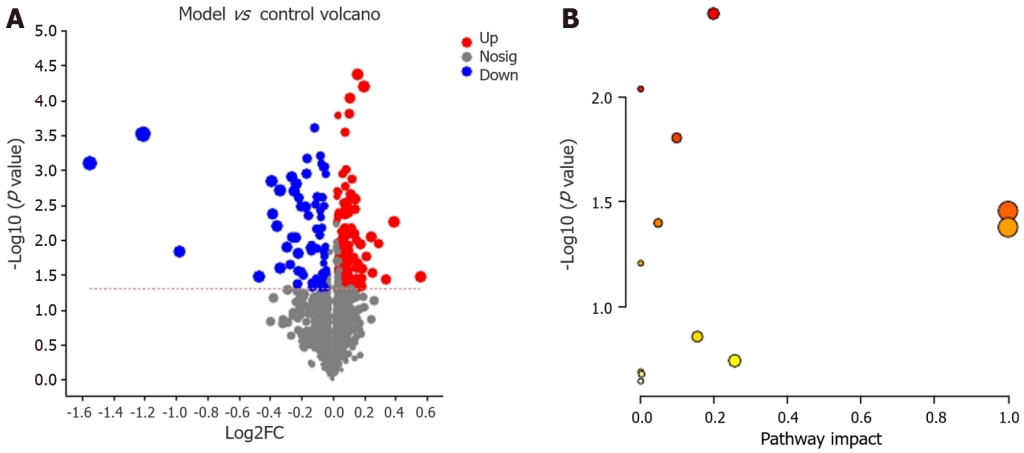

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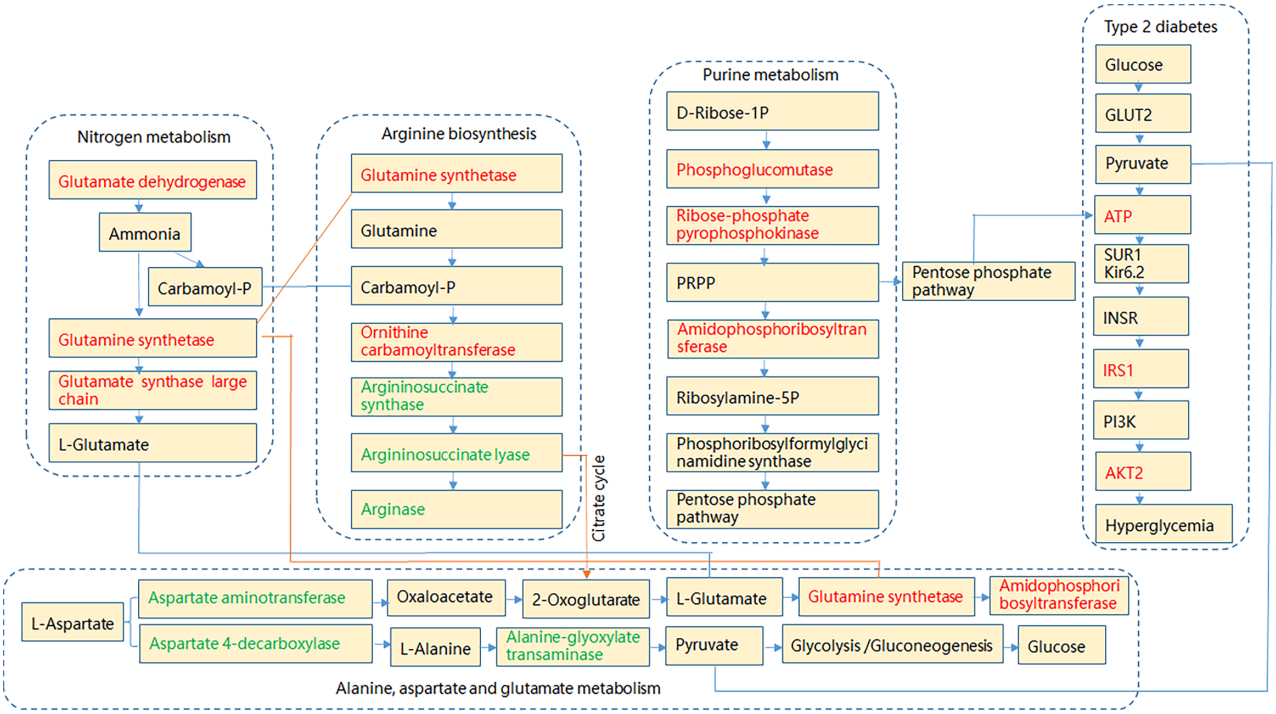
Figure 6 Differential metabolites and metabolic pathways. A: Volcano plot of the differential positive- and negative-ion metabolites; B: Metabolic pathways showing differential regulation in Goto Kakizaki rats.

A clear separation among the communities was observed between the control and model groups, which suggested that the gut microbiota shows substantial differences between the diabetes and nondiabetes groups. At the phylum level, Bacteroidetes and Firmicutes were dominant in GK and Wistar rats, with proportions greater than 80%. Although no significant differences were detected, a higher relative abundance of Firmicutes and a lower relative abundance of Bacteroidetes were found in the model group than in the control group. These findings may be related to the fact that the rats were fed breeding feed after weaning. These results may also be related to the shorter disease course in the T2DM rats because the gut microbiota is seriously disturbed, and the development of characteristic hyperglycaemia lags behind.



DOI: 10.4239/wjd.v14.i3.255

Figure 7 Potential targets of the metabolic pathways showing differential regulation in Goto Kakizaki rats. Compared with the control group, green and orange represent decreased and increased levels, respectively.

At the species level, a higher abundance of *Allobaculum stercoricanis* and lower abundances of *Prevotella* sp. CAG:604 and *Lactobacillus murinus* were found in the model group compared with the control group. *Allobaculum stercoricanis* belongs to the *Allobaculum* genus (Firmicutes), for which a significant difference was found at the genus level. A previous study showed that *Allobaculum* presented a 10.95-fold increase in HF diabetic mice compared with ND-fed mice[25], and the corresponding increase found in this study was 2.75-fold. In a previous study, *Allobaculum* was identified as an important biomarker of metabolic disorders, particularly diabetes[26,27]. This study also showed that *Allobaculum stercoricanis* was closely related to FBG, HOMA-IR index and a decrease in HOMA-β index. Different individual animals, even within the same species, show different feed conversion efficiencies. Animals with a high feed conversion efficiency show higher mRNA expression levels of insulin-like growth factor[28], which can further regulate glucose. The abundance of *Prevotella* sp. CAG:604 is related to the feed conversion efficiency[29]. This study revealed a decrease in the abundance of *Prevotella* sp. CAG:604, which may be one of the reasons for the diabetic phenotype of GK rats, and a direct negative correlation was found between these factors. *Prevotella* sp. CAG:604 could be considered a potential biomarker of diabetes. *Lactobacillus murinus* was decreased in GK rats, and this finding is consistent with the results reported by Cui *et al*[30] and Yue *et al*[31]. With increases in the abundance of *Lactobacillus murinus*, the glucose level and HOMA-IR index showed a downwards trend. *Lactobacillus murinus* is considered a beneficial gut microbe[32]. In a previous study, a probiotic group whose members drank *Lactobacillus casei* strain milk showed a significant increase in the faecal count of *Lactobacillus*. The condition of patients with T2DM has also been controlled[33]. We speculate that drinking milk containing *Lactobacillus murinus* may also control the glucose levels.

The OPLS-DA model suggested that the positive- and negative-ion metabolic profiles differed between the GK and Wistar rat faeces. Fourteen potential metabolic biomarkers were assigned to bile acids, benzenoids, amino acids, steroids, amines, pyridine carboxylic acids and derivatives, and fatty acids. The topological analysis of the metabolic pathways showed that the difference between GK and Wistar rats was mainly due to arginine biosynthesis; primary bile acid biosynthesis; alanine, aspartate and glutamate metabolism; purine metabolism; linoleic acid metabolism; and nitrogen metabolism. These results were consistent with those found in previous studies[34,35]. The metabolic pathway analysis showed that the differences were mainly due to amino acid metabolism, lipid metabolism, nucleotide metabolism, and energy metabolism. These pathways provide ideas for future research, and the results show that in addition to abnormal carbohydrate pathways, fat and protein are also metabolized in a disorderly manner leading to dysfunctions of the eyes, kidneys, blood vessels, and nerves, among other organs.

We observed marked decreases in the excretion of glycochenodeoxycholic acid and glycocholic acid in rat faeces, as reported by Sun *et al*[36]. The levels of glycochenodeoxycholic acid are increased in the liver, blood and ileum[36-38]. Lu *et al*[39] showed that increased plasma glycochenodeoxycholic acid

levels are associated with an increased risk of T2DM. The relationship among 3-α, 7-α, 26-trihydroxy-5-β-cholestane and T2DM has been less well studied, and we found that 3-α, 7-α, 26-trihydroxy-5-β-cholestane was positively related to FBG and HOMA-IR, which may be a research topic that will be addressed in the future. Hyperuricaemia is particularly common in patients with T2DM[40]. Some clinical studies have observed that ursodeoxycholic acid, as the representative drug regulating bile acid metabolism, exerts a certain effect on reducing glucose, glycosylated haemoglobin A1c, and weight and increasing GLP-1 secretion[41,42]. Although ursodeoxycholic acid is not widely used in clinical practice, it may be used as a potential drug for the treatment of T2DM. In addition to excretion in the kidneys, uric acid can also be secreted into the intestine and metabolized by microorganisms[43]. This interaction could potentially modulate the serum uric acid levels[44]. This study revealed an increase in the intestinal excretion of uric acid, indicating that uric acid in the body is likely to be a risk factor for T2DM and could aggravate this condition. In our study, we also found that uric acid was positively related to FBG and HOMA-IR.

In the presence of glutathione, a series of physiological reactions result in a low antioxidative defence capacity. β cells gradually die, and progression to T2DM occurs[45]. This study showed that the glutathione level was increased in the GK group and was positively related to the FBG and HOMA-IR. Hyperglycaemia is a potential risk factor for the N-acetylaspartate network[46]. The present study also identified this phenomenon. The N-acetylaspartate level was higher in GK rats. A high level of N-acetyl-L-glutamic acid may result in increased blood pressure[47]. Uncontrolled blood pressure can significantly increase the risk of macroangiopathy in diabetes. The relationship of allantoic acid with diabetes has not been studied, but the present study revealed that allantoic acid was positively related to FBG and HOMA-IR. At baseline, a previous study showed that amino acids are upregulated in T2DM patients. Dysregulation of amino acid metabolism may occur earlier than glucose metabolism in T2DM [48]. This finding suggests that patients with T2DM should not consume a diet with a high protein level.

We found that the concentration of sphinganine was increased in GK rats. Sphinganine is positively related to *Lachnospiraceae bacteria*, *Allobaculum stercoricanis*, and *Lactobacillus johnsonii* and is negatively related to *Prevotella* sp. CAG:604 and *Phascolarctobacterium succinatutens*. Studies have shown that when obese people lose weight, they exhibit an increase in the abundance of *Phascolarctobacterium succinatutens* [49]. Weight loss is conducive to glucose control. Sphinganine is formed from ceramide and is a risk factor for β-cell dysfunction in T2DM[50]. Under these conditions, insulin secretion was observed to be reduced, and the inhibition of ceramide metabolism was weakened. An increase in 13(S)-HODE has been observed in individuals with nonalcoholic fatty liver disease[51]. Because the common pathogenesis of nonalcoholic fatty liver and diabetes is insulin resistance, diabetes may also be accompanied by elevated levels of 13(S)-HODE, and our research confirms this hypothesis. 13(S)-HODE was found to be positively related to FBG and HOMA-IR, and *Phascolarctobacterium succinatutens* was enriched in the model group.

We observed that the linoleic acid level was elevated in GK rats. Linoleic acid levels are markedly higher among populations with a higher prevalence of T2DM[52]. Linoleic acid is positively related to *Allobaculum stercoricanis* and is negatively related to *Prevotella* sp. CAG:604. 4-Pyridoxic acid is a catabolite of vitamin B6 metabolism, and our study showed that the 4-pyridoxic acid levels were increased in the model group and positively related to FBG and HOMA-IR. A higher level of vitamin B6 is associated with a higher HOMA-IR values and T2DM[53]. Furthermore, the risk of all-cause mortality in patients with T2DM is higher among those with 4-pyridoxic acid levels in the highest quartile[54]. 2-Methyl-3-hydroxy-5-formylpyridine-4-carboxylate is also a catabolite of vitamin B6 metabolism, and a reduction in its content can help regulate the glucose levels[55,56]. This compound was was detected at to be negatively related to *Prevotella* sp. CAG:604 and *Dorea* sp. CAG:317 and showed increased levels in the model group in this study. The results indicated that the glucose level was elevated in GK rats and that chenodeoxycholic acid glycine conjugate was negatively correlated with FBG and HOMA-IR, which was consistent with the results reported by Zhang *et al*[57].

Feng *et al*[58] found that glutamine synthetase activity was significantly increased after the induction of diabetes by streptozotocin in rats. The serum levels of ornithine carbamoyltransferase are increased in KK-Ay diabetic mice[59]. The liver is involved in glucose metabolism. In a state of hyperglycaemia, the liver is damaged to varying degrees, resulting in the abnormal expression of some proteases. Phosphoglucomutase is a key protease in carbohydrate metabolism[60]. In particular, this protease is closely related to obesity. Endothelial cell function damage mostly occurs in T2DM and is related to argininosuccinate synthase and argininosuccinate lyase. In particular, TNF-α downregulates argininosuccinate synthase expression[61,62]. The activity of amidophosphoribosyltransferase is higher in the kidneys of diabetic rats[63]. The results indicate that amidophosphoribosyltransferase is related to the accretion of nucleic acids. A low level of aspartate aminotransferase is an independent risk factor for frail T2DM patients[64]. Glycogen is synthesized in the liver, and changes in a series of liver enzymes affect the synthesis of glycogen. For example, aspartate 4-decarboxylase and alanine-glyoxylate transaminase were detected in this study. The occurrence of T2DM is related to many signalling pathways. Pyruvate, which is the end product of glycolysis, is transported into mitochondria and drives ATP production[65]. This study showed that ATP imbalance was the main reason for insulin resistance and thus mainly affected the IRS1 and AKT2 potential in the insulin resistance signalling pathway.

We identified different gut microbes and metabolites in GK rats, and the results provide new research directions related to faecal bacteria transplantation, supplementary metabolites, and drug screening, among other topics. This study also has some limitations, such as a lack of drug interventions for verification and a lack of long-term observations of the dynamic changes in gut microbes and metabolites. In future research, we will further improve the design scheme for studying metabolic disorders through the influence of nitrogen metabolism, arginine biosynthesis, primary bile acid biosynthesis, purine metabolism, alanine, aspartate and glutamate metabolism and insulin resistance, which can lead to the emergence of T2DM. In addition to the characteristics of hyperglycaemia, disordered fat and protein levels are also observed in patients with T2DM. T2DM is a clinical syndrome with multiple causes, and we thus believe that the treatment strategy for T2DM should be comprehensive and focus on glucose, lipids, protein, weight and other factors. This finding may provide ideas for the future management of patients with T2DM.

CONCLUSION

The present study revealed that disordered compositions of gut microbes and metabolites are putative common defects observed in GK rats by metagenomics and untargeted metabolomics. T2DM-related changes in gut microbes and metabolites may contribute to hyperglycaemia. However, further experiments are needed to summarize the different stages of T2DM.

ARTICLE HIGHLIGHTS

Research background

Goto Kakizaki (GK) rats share common features with human type 2 diabetes (T2DM). Therefore, the results obtained from analyses of the gut microbiota and metabolites of GK rats will be more similar to those of patients with T2DM.

Research motivation

The gut microbiota and metabolites are critical in T2DM. Therefore, alterations in different gut microbiota and metabolites may provide useful evidence for analysing the pathogenesis and treatment of T2DM.

Research objectives

To investigate the alterations in gut microbiota and metabolites in the faeces of T2DM rats.

Research methods

Systematic characterization of the faecal gut microbes and metabolites of GK rats using metagenomic and untargeted metabolomic approaches and analysis of the relationship between gut microbes and metabolites under conditions of glucose and insulin resistance.

Research results

The GK rats displayed significant differences in the gut microbiota structure compared with the control group. The results demonstrated that the GK rats presented significantly decreased abundances of *Prevotella* sp. CAG:604 and *Lactobacillus murinus* ($P < 0.05$) and a significantly higher abundance of *Allobaculum stercoricanis* ($P < 0.01$). Orthogonal partial least squares discriminant analysis suggested that the faecal metabolic profiles differed between the GK and control groups. Fourteen potential metabolic biomarkers, including glycochenodeoxycholic acid, uric acid, 13(S)-hydroxyoctadecadienoic acid, N-acetylaspartate, β-sitostenone, sphinganine, 4-pyridoxic acid, and linoleic acid, were identified. The metabolic pathways showing the main differences were arginine biosynthesis; primary bile acid biosynthesis; purine metabolism; linoleic acid metabolism; alanine, aspartate and glutamate metabolism; and nitrogen metabolism.

Research conclusions

The present study revealed that disordered compositions of gut microbes and metabolites are putative common defects observed in GK rats by metagenomics and untargeted metabolomics.

Research perspectives

Gut microbes and metabolites play a key role in carbohydrate metabolic pathways. Therefore, an evaluation of the involvement of dynamic changes in gut microbes and metabolites may be important in the future.

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FOOTNOTES

Author contributions: Zhao JD and Fang ZH participated in the design of the study and wrote the manuscript; Sun M, Li Y, Yu CJ, Cheng RD, Wang SH and Du X performed the experiment and helped complete the data analysis; The final version of the manuscript was reviewed and approved by all the authors.

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Data sharing statement: The data used to support the findings of this study are included within the article.

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ORCID number: Jin-Dong Zhao 0000-0003-0005-1820; Min Sun 0000-0002-8528-0050; Yan Li 0000-0002-7640-7216; Chan-Juan Yu 0000-0002- 4897-4109; Ruo-Dong Cheng 0000-0002-0861-7748; Si-Hai Wang 0000-0003- 3526-7867; Xue Du 0000-0002-6573-8060; Zhao-Hui Fang 0000-0002-3197-1534.

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