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# Pharmacogenomics and pharmacomicrobiomics in type 2 diabetes mellitus (T2DM)

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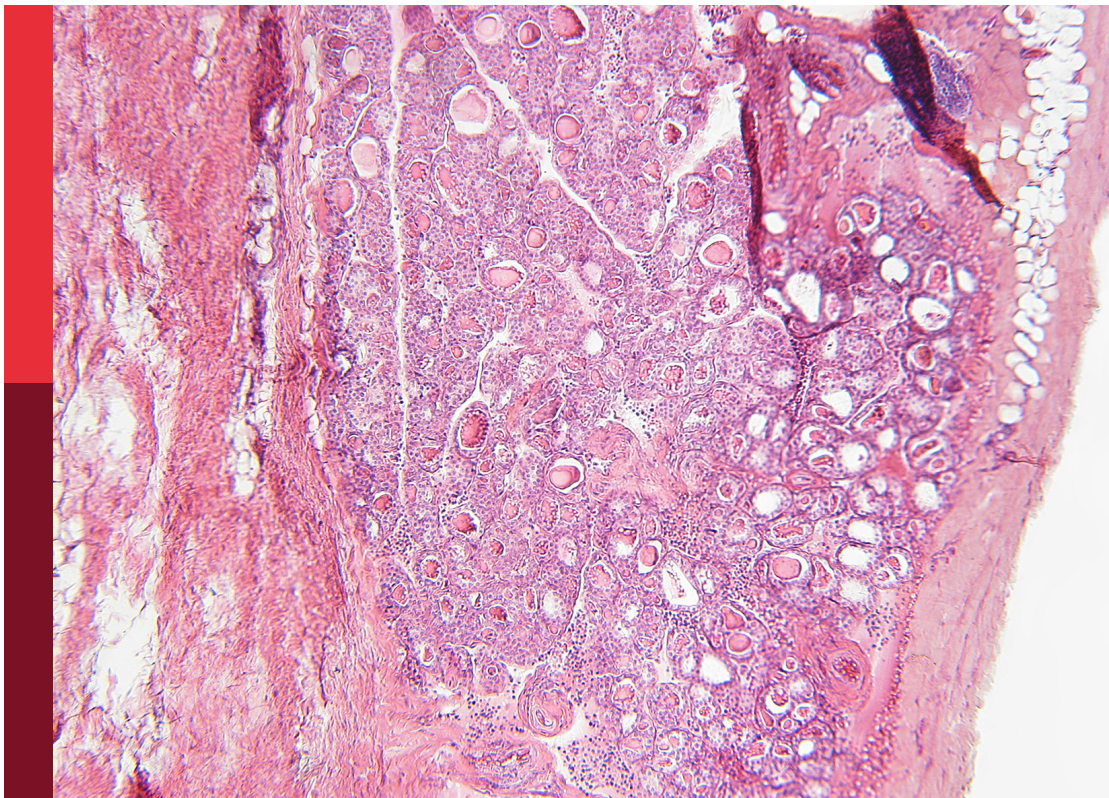

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# Pharmacogenomics and pharmacomicrobiomics in type 2 diabetes mellitus (T2DM)

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# Editorial: Pharmacogenomics and pharmacomicrobiomics in type 2 diabetes mellitus (T2DM)

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**pharmacogenomics, pharmacomicrobiomics, polymorphisms, microbiome, type 2 diabetes mellitus, precision medicine**

Editorial on the Research Topic
Pharmacogenomics and pharmacomicrobiomics in type 2 diabetes mellitus (T2DM)

Type 2 Diabetes mellitus (T2DM) is a complex and multifactorial metabolic disorder, caused by an interplay of genetic variations, unhealthy lifestyles and environmental factors. For many years, pharmacogenomics contributed to a great step towards precision medicine (1). The evidences from pharmacogenomics have revolutionized our understanding of the role of genetic variations in the T2DM pathogenesis and therapeutic response to antidiabetic drugs. In recent years, pharmacomicrobiomics, an extension of pharmacogenomics, has also provided unique insights into personalized medicine by investigating the interaction between drugs, host and gut microbiota (2). Therefore, we organized this Research Topic that aimed to shed light on the recent progress in pharmacogenomics and the emerging fields of pharmacomicrobiomics for T2DM.

In a series of contributions, multiple review articles have focused on the pharmacomicrobiomics for T2DM. The imbalance of human gut microbiota and their metabolites are increasingly considered to play a critical role in the development of T2DM and treatment outcomes of antidiabetic drugs. The review article of Chu et al. summarized the differences in the composition of gut microbiota between patients with T2DM and healthy individuals. Importantly, it also provided the current evidence on pharmacomicrobiomics of Western Medicine (WM) and Traditional Chinese Medicine (TCM) in T2DM. The effects of both WM and TCM could increase the relative abundance of health promoting bacteria, such as Akkermansia muciniphila, Blautia, and Bifidobacterium adolescent. Additionally, TCM might complement the efficacy of WM through alteration of microbiota. This review article of Wu et al. summarized the interaction between gut microbiota and its metabolites, including short-chain fatty acids, lipopolysaccharide, bile acids, trimethylamine-N-oxide, tryptophan and indole derivatives, and their role in the pathogenesis of T2DM. In addition, they discussed the potential

strategies for prevention and treatment of T2DM by modulating the gut microbiota and its metabolites. The approaches included the use of probiotics, prebiotics, synbiotics, fecal microbiota transplantation, dietary interventions, bacteriophages, microbiota-targeted drugs and postbiotics. The review from Jia et al. highlighted the microbe-drug-host interactions, in particular for the antidiabetic drugs including metformin, thiazolidinedione, α-glucosidase inhibitors, sodium-glucose cotransporter 2 inhibitors, glucagon-like peptide-1 receptor agonists, dipeptidyl peptidase-4 inhibitors and traditional Chinese medicine. They also discussed the value of pharmacomicrobiomics findings as innovative potential personalized treatments for T2DM.

Post-transplant diabetes mellitus (PTDM) is a common and deleterious co-morbidity after solid organ transplantation. Intestinal dysbiosis may play a key role in the pathophysiology of drug-induced hyperglycaemia and diabetes mellitus (3). Based on complementary and coherent scientific evidence, the perspective article of Faucher et al. discussed the potential association between intestinal dysbiosis and PTDM, and provided arguments for the value of monitoring the microbiota diversity and function in solid organ transplantation.

Impaired glucose tolerance (IGT) is a necessary process for developing T2DM and an important stage where T2DM can be controlled and reversed. Thus, effective interventions are urgently needed (4). The original research of Guo et al. investigated the potential therapeutic targets of liraglutide in treatment of streptozotocin-induced impaired glucose tolerance (IGT) rats. Based on the tandem mass tag technique, the results revealed the target proteins of liraglutide, such as TBC1D13, PPIF, MPRIP, ME2, CYP51, DAD1, PTPA, TXNL1, ATG2B, BCL-2, etc. in the treatment of IGT. The clinical trial conducted by Yan et al. assessed the effect of early probiotic intervention in preventing conversion of patients with IGT to T2DM in the Probiotics Prevention Diabetes Program trial with follow up for 6 years. The patients with IGT were randomly assigned to either placebo treatment or probiotic supplementation with Bifidobacterium, Lactobacillus acidophilus and Enterococcus faecalis. After 6 years follow up, although active probiotic supplementation was safe, no significant difference was found in the cumulative incidence of developing T2DM (59.1% with probiotic treatment versus 54.5% with placebo).

Significant strides in the management of diabetic kidney disease (DKD) have evolved in parallel with the growing knowledge about its pathophysiological mechanisms (5). The original research of Wu et al. investigated the effects of dapagliflozin on DKD and gut microbiota composition during the progression of diabetes, performed 16S rRNA gene sequencing on fecal samples from C57BL/6 mice administrated with physiological saline, db/db mice administrated with physiological saline, and db/db mice treated with dapagliflozin at three timepoints of 14 weeks,18 weeks and 22 weeks. Based on their results, the authors highlighted the dynamic improvement of the gut microbiota over time accounting for the protective effect of dapagliflozin on DKD.

With regarding to the recent progress in pharmacogenomics T2DM, the Research Topic comprises two articles. The original research conducted by Song et al. evaluated the potential impact of two *PPARD* genetic variants (rs2016520 and rs3777744) on the therapeutic responses to exenatide in treating Chinese patients with T2DM.The study showed patients with *PPARD* rs2016520 TT genotype or rs3777744 G allele may have a poor response to exenatide therapy. One interesting point in this study is that *PPARD* rs2016520 and rs3777744 showed dramatically different allele frequencies in different ethnic populations. So, future studies are needed to explore the effects of *PPARD* rs2016520 and rs3777744 in the therapeutic responses to exenatide among other racial populations (6). The interactions of gene-lifestyle or gene-environment on the risk of T2DM is another important topic worthy of intensive investigation. Based on the data from the Korean Genome and Epidemiology Study Cohort, the original research article of Apio et al. investigated the association between dietary patterns and T2DM and conducted a gene-diet interaction analysis related to T2DM. The results showed that dietary pattern of poor amounts of antioxidant nutrients conferred to the risk of T2DM. The gene-diet interaction analysis indicated that dietary patterns affected pathway mechanisms in the development of T2DM.

In summary, the published articles in this Research Topic covered interesting findings and various aspects of the Research Topic, providing significant insights into the fields of pharmacogenomics and pharmacomicrobiomics in T2DM. In the future, the integration of pharmacogenomics and pharmacomicrobiomics will hold great promise for advancing personalized medicine of T2DM.

## Author contributions

JL: Conceptualization, Writing – original draft, Writing – review & editing, Project administration, Supervision. YS: Conceptualization, Supervision, Writing – review & editing, Project administration. WZ: Conceptualization, Project administration, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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REVIEW
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# Pharmacomicrobiomics in Western Medicine and Traditional Chinese Medicine in Type 2 Diabetes

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Pharmacomicrobiomics refers to the interactions between foreign compounds and the gut microbiome resulting in heterogeneous efficacy, side effects, and toxicity of the compound concerned. Glucose lowering drugs reduce blood glucose by modulating insulin secretion and its actions as well as redistributing energy disposal. Apart from genetic, ecological, and lifestyle factors, maintaining an equilibrium of the whole gut microbiome has been shown to improve human health. Microbial fingerprinting using faecal samples indicated an 'invisible phenotype' due to different compositions of microbiota which might orchestrate the interactions between patients' phenotypes and their responses to glucose-lowering drugs. In this article, we summarize the current evidence on differences in composition of gut microbiota between individuals with type 2 diabetes (T2D) and healthy individuals, the disruption of the balance of beneficial and pathogenic microbiota was shown in patients with T2D and how Western Medicine (WM) and Traditional Chinese Medicine (TCM) might re-shape the gut microbiota with benefits to the host immunity and metabolic health. We particularly highlighted the effects of both WM and TCM increase the relative abundance of health promoting bacteria, such as, *Akkermansia muciniphila*, *Blautia, and Bifidobacterium adolescentis*, and which have been implicated in type 2 diabetes (T2D). Several lines of evidence suggested that TCM might complement the efficacy of WM through alteration of microbiota which warrants further investigation in our pursuit of prevention and control of T2D.

**Keywords: medication, traditional Chinese medicine, diabetes, microbiota, *Akkermansia***

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## INTRODUCTION

Type 2 diabetes (T2D) and its complications constitute a worldwide public health challenge. In 2020, it was estimated that 537 million people had diabetes with the majority residing in low- and middle-income countries (1). Over 95% of affected people have T2D which is associated with an increased risk of premature death and multiple morbidities. Type 2 diabetes is a complex disease due to multiple risk

factors where delayed diagnosis and intervention can lead to widespread micro and macrovascular complications. The distribution of gut microbiota in is disrupted in patients with type 2 diabetes and cardiovascular disease. Butyrate producing organisms such as *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, and *Akkermansia* were negatively associated with T2D, while *Ruminococcus*, *Fusobacterium*, and *Blautia* were more abundant in T2D patients. Gut microbiota can also influence other cardiometabolic risk factors, such as hypercholesteoleremia by modulating metabolite production of bile acids, coprostanol and short chain fatty acids (SCFAs) (2). The balance of beneficial and pathogenic bacteria may linked to different diseases (3). Apart from personal suffering, these complications are associated with enormous healthcare costs and loss of societal productivity, calling for more accurate diagnosis and efficient prevention and control strategies (1).

Rapid urbanization is associated with multidimensional changes in our ecosystem including but is not limited to mechanization, food technology, physical space, cultures, jobs, and leisure which greatly influence our lifestyles notably diet and exercise (4). These changes in the macroenvironment can affect the host internal milieu which can be further influenced by the microorganisms in their gut, referred to as microbiota (2). With the advent of sequencing technology, the collective genomes of these microbiota (microbiome) can be defined and categorized. In recent years, many studies have reported associations of the development of T2D with changes in the gut microbiome (5, 6). Possible mechanisms include insulin resistance, changes in pH and bowel permeability (7), endotoxemia (8) as well as changes in the metabolism of bile acids (9) and short-chain fatty acids (SCFA) (10, 11).

In support of the possible causative roles of microbiota in the pathogenesis of T2D, there are also reports suggesting that some Western Medicine (WM) and Traditional Chinese medicine (TCM) mediated their effects through changes in the microbiome (12). In this review, we summarize differences in the composition of gut microbiota between healthy individuals and patients with T2D as well as the effects of different WM and TCM on gut microbiota, which act in concert with lifestyle factors to orchestrate the diversity of the whole gut microbiome and influence metabolic health.

## DIFFERENCES OF GUT MICROBIOTA IN HEALTHY INDIVIDUALS AND PATIENTS WITH T2D

Microbial fingerprinting refers to the use of fecal samples to identify the unique pattern of the microbiome, referred to as 'dysbiosis', associated with a disease phenotype. In this section, we reviewed published data on the pattern of microbiota in T2D and explored the possible effects of different medications in altering microbiota homeostasis.

In a recent systematic review, patients with T2D had a higher abundance of *Lactobacilli* and a lower abundance of *Bifidobacteria* than healthy individuals (5). In this analysis which included 13 case-control studies including 575 patients with T2D and 840 healthy controls, the authors reported that these T2D-associated microbiome might be further influenced by the effect of different medications. In another study involving 11 newly diagnosed patients with T2D, researchers compared their microbiota with that of 17 individuals with prediabetes and 39 patients with established T2D. Compared to healthy individuals, newly diagnosed T2D had a lower abundance of *Akkermansia*, *Blautia*, *Ruminococcus* (13),*Clostridium leptum, and Clostridium coccoide* (14), but these changes were restored in patients with T2D on antidiabetic treatment (15). *C. leptum* and *C. coccoides* are butyrate-producing bacteria and are inversely related to glucose and homeostatic model assessment (14).

Several lines of evidence indicated that intestinal microbial overgrowth was found in patients with newly diagnosed T2D compared with individuals with normal glucose tolerance (NGT). While individuals with impaired glucose tolerance (IGT) and T2D had similar patterns of dysbiosis, this was not found in those with impaired fasting glucose (IFG) (16). In a 4-year study involving individuals with prediabetes, researchers reported plasma glucose was negatively associated with *Odoribacter*, *Oscillibacter*, and *Pseudoflavonifracter* (15).

*Clostridium leptum* and *C. coccoides* were microbiota that could influence human health by altering intestinal peristalsis, promoting synthesis of vitamins, promoting excretion of harmful substances, and protecting the gut from an invasion of pathogens. In treatment-naïve patients with T2D, there was relative depletion of *C. coccoides* and *C. leptum* considered to be health-promoting microbiota. In these patients, the microbiota was also dominated by harmful microbiota, such as *Escherichia/Shigella* (17). Other species implicated in T2D included *Akkermansia, Blautia, Clostridium* spp., and *Ruminococcus.* Of note, low abundance of *Akkermansia muciniphila* had been associated with obesity and aging while its administration had been shown to increase the intestinal levels of endocannabinoids with reduced inflammation (18). Recently, some species in the genera *Clostridium* and *Ruminococcus* had been reclassified as *Blautia*, the latter being a newly discovered anaerobic probiotic which was negatively correlated with metabolic diseases such as T2D, obesity, and fatty liver (19). All these studies found a decrease in the number of butyrate-producing bacteria, such as *Akkermansia, Blautia*, and *Bifidobacteria*, and an increase in conditional pathogens, *Escherichia/Shigella.*

## EFFECTS OF WM ON THE GUT MICROBIOME IN TYPE 2 DIABETES

**Table 1** summarizes the effects of WM on the composition of the microbiota. Biguanide (e.g., metformin) is the most popular oral glucose-lowering drug often used as first-line therapy in patients with T2D. Metformin has pluripotent effects which improve energy metabolism and reduce inflammation. By inhibiting the mitochondrial complex I as a key component of the electron transport system, metformin activates AMPK (adenosine 5′-

**TABLE 1** | Summary of the effects of western medicine on the composition of the gut microbiome in T2D patients.

| Drugs | Author (Years) | Patients | Periods | Study design | Effect of treatment on microbes | Additional remarks |
|---|---|---|---|---|---|---|
| Metformin | Wang et al., 2018 (20) | 37 patients with T2D (18 treated with metformin and 19 treated with GLP-1 mimetics) | 6 weeks | Cross sectional study | Metformin ↑*Sutterella* | A higher abundance of *Akkermansia* in patients with short and medium duration than those with long duration of diabetes |
| Metformin | Sun et al., 2018 (11) | 22 patients with newly diagnosed T2D treated with metformin | 3 days | Intervention study | Metformin ↑*Lactobacillus sanfranciscensis* ↓*Bacteroides fragilis* | Metformin improves obesity-induced glucose intolerance and insulin resistance through the gut microbiota |
| Metformin | Wu et al., 2017 (21) | 40 patients with newly diagnosed T2D treated with metformin | 4-6 months | Randomized placebo controlled crossover study | Metformin ↑*Akkermansia muciniphila, Bifidobacterium adolescentis, Lactobacillus fermentium, Peptoniphilus* sp. *Ruminococcus* sp., *etc.* ↓*Intesinibacter bartlettii, and Clostridium* spp. | Decrease in HbA1c and fasting plasma glucose after the metformin treatment |
| Metformin | Cuesta-Zuluaga et al., 2017 (22) | 28 patients with T2D (14 treated with metformin and 14 not-treated with metformin) and 84 without diabetes | Not mentioned | Cross-sectional case-control study | Metformin ↑*Akkermansia muciniphila, Butyrivibrio, Bifidobacterium bifidum, Megasphaera*, and *Prevotella* | There were significant differences in the comparison in β diversity of microbiome between metformin and non-metformin users |
| Acarbose | Gu et al., 2017 (23) | 94 patients with newly diagnosed T2D treated with acarbose or glipizide | 3 months | Multicentre parallel comparison | Acarbose ↑ *Lactobacillus* and *Bifidobacterium* ↓*Bacteroides* | Reductions in HbA1c, fasting, and postprandial plasma glucose in both treatment arms |
| Acarbose | Su et al., 2015 (24) | 59 patients with T2D patients treated with acarbose 36 patients treated with other glucose-lowering drugs 55 healthy controls | 4 weeks | Cross-sectional case-control study | Acarbose ↑*Bifidobacterium longum* | Acarbose significantly reduced lipopolysaccharides and prothrombin activator inhibitor-1 |
| GLP-1 mimetics | Shang et al., 2021 (25) | 40 patients with T2D switched from metformin to liraglutide | 4 months | Observational study | Liraglutide ↑*Collinsella*, *Akkermansia*, and *Clostridium* | BMI, HbA1c, homeostasis model assessment of insulin resistance (HOMA-IR), fasting blood glucose, 2-hour postprandial blood glucose, and lipid profiles were significantly lower after liraglutide-treatment |
| GLP-1 mimetics | Wang et al., 2018 (20) | 37 patients with T2D (18 treated with metformin and 19 treated with GLP-1 mimetics) | 6 weeks | Cross-sectional study | GLP1 ↑*Akkermansia* | Patients receiving a GLP-1 agonist had higher *Akkermansia* abundances than those on metformin. |
| SGLT2i and Gliclazide | Bommel et al., 2019 (26) | 44 metformin-treated patients with T2D randomized to either dapagliflozin or gliclazide | 12 weeks | Randomized double-blind, comparator-controlled, parallel-group trial | No change in microbiota with either dapagliflozin or gliclazide treatment | Both drugs improved glycaemic control with dapagliflozin reducing and gliclazide increasing fasting plasma insulin. |

*GLP-1, Glucagon-like peptide-1.*

monophosphate-activated protein kinase) resulting in reduced anabolism and increased catabolism (27). Metformin also reduces hepatic glucose production and absorption of glucose from the intestine. Additionally, the gut microbiota has been linked to the glucose-lowering efficacy and tolerance with metformin.

In an animal study, 28 high-fat-fed mice were randomized to the control and metformin group equally, metformin group increased the abundance of genus *Bacteroides, Akkermansia, Parabacteroides, Christensenella, Clostridales*, and decreased the abundance of genus *Muribaculum, Lachnoclostridium, Coprococcus, Dorea, Papillibacter, Oscillospira, Ruminococcus*, and *Desulfovibrio* in 12 week treatment (28). In other animal studies, metformin has been shown to constantly promote of the abundance of *Akkermansia* at a dose ranging from 75 to 300mg/kg/d given for 4 days to 14 weeks in ten controlled studies (29–38). In a 4-month double-blind, placebo-controlled study involving 40 patients with T2D, who were treated with metformin showed no differences in body weight, body fat, and fasting plasma insulin but reduced glycated hemoglobin (HbA1c) and fasting plasma glucose. Treatment with metformin also increased *Akkermansia muciniphila*, *Bifidobacterium adolescentis, Lactobacillus fermentium, Peptoniphilus* sp. *Ruminococcus* sp. *Cronobacter turicensis, Enterobacter lignolyticus, Citrobacter koseri, Yersinia enterocolitica subsp., Klebsiella pneumonia, Enterobacter asburiae, Enterobacter*

*cloacae subsp*, and decreased *Intestinibacter bartlettii, Clostridium beijerinckii, Clostridium* sp. *Clostridium perfringens, Clostridium botulinum*, and *Clostridium butyricum*. Notably, *Bifidobacterium adolescentis* was the only probiotic that exhibited a dose-related response to metformin in the gut microbiome. In animal and human studies, metformin increased the abundance of *Akkermansia muciniphila* (39) but inconsistent in other health promoting microbiota, such as *Blautia (*40), *Prevotella (*41), and *Roseburia* (38). The results in humans are different from the results of animal studies because of differences in intestinal microbiome between humans and animals (42) affecting by eating habit, physical activities, ethnic origins, course of disease, comorbidities, and multiple medications.

In another two clinical trials, metformin also increased *Akkermansia muciniphila* and SCFA-producing microbes (10) including *Butyrivibrio, Bifidobacterium bifidum, Megasphaera*, and *Prevotella* (22). These microbes utilized multiple dietary substrates to produce an array of metabolites. The abundance of *Bifidobacterium* species can activate multiple genes involved in carbohydrate metabolism (43) and *Prevotella* species contribute to starch degradation (22). In another study involving patients with newly diagnosed T2D, 3-day treatment with metformin decreased the genus *Bacteroides* with increased bile acid glycoursodeoxycholic acid (GUDCA) accompanied by reduced hyperglycemia. In mice, colonization of *B. fragilis* abrogated the glucose-lowering and GUDCA increasing effects of metformin suggesting that this microbe might play a mediating role in these metabolic effects of metformin (11).

Acarbose is an alpha-glucosidase inhibitor. It is a highly popular glucose-lowering drug in China (44) and many Asian countries (45). This WM is a complex molecule that inhibits the conversion of disaccharides to monosaccharides and thus converts carbohydrates into a fiber-like molecule. This leads to an increased amount of indigestible carbohydrates in the lower part of the intestine available for fermentation by microbiota. In an animal study, compared to the control and a low dose acarbose (25 ppm), a high dose of acarbose (400 ppm) promoted the abundance of *Bacteroidaceae* and *Bifidobacteriaceae* and decreased in the abundance of *Bacteroidales S24-7* and species *Akkermansia muciniphila* under the controlled diet (46). However, in another animal study when compared to placebo, acarbose displayed a higher abundance of *Ruminococcus 2* and *Lactobacillus* and decrease the species *Akkermansia muciniphila (*47). In a human study, after treatment with acarbose, *Lactobacillus* and *Bifidobacterium* species thrived with depletion of the original gut microbiota including *Bacteroides*, *Alistipes*, and *Clostridium* (23). In a clinical study, acarbose was found to increase the abundance of *Bifidobacterium* and *Lactobacillus*, which correlated inversely with changes in HbA1c and body weight. At the genus level, acarbose decreased the abundance of *Bacteroides* and at a species level, *Bacteroides plebeius, Bacteroides dorei/vulgatus*, and *Clostridium bolteae*. In a randomized trial, acarbose treatment increased the abundance of *Bifidobacterium longum* and *Enterococcus faecalis* in patients with T2D. However, these results might have been confounded by its co-administration with metformin and other glucose-lowering drugs (24) which could also alter the diversity of microbiota. In people with prediabetes (48), compared to placebo, treatment with acarbose increased abundance of *Lactobacillus* and *Dialister* and reduced abundance of *Butyricicoccus*, *Phascolarctobacterium*, and *Ruminococcus*. However, the study did not differentiate between IFG and IGT. Such differentiation is important given that the microbiome in the IGT group is more akin to that in individuals with T2D (16).

Dipeptidyl peptidase (DPP-4) inhibitors include sitagliptin, saxagliptin, linagliptin, and alogliptin. This drug class prevents the enzymatic degradation of glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2). Glucagon-like peptide (GLP) and glucose-dependent insulinotropic polypeptide (GIP) are incretins or peptides secreted by the enterocytes in the gut. Incretins are natural hormones that suppress glucagon and hepatic glucose production whilst augmenting insulin secretion during meal time resulting in reduced fasting and post-prandial blood glucose. In animal studies, DPP4-inhibitors reduced the abundance and diversity of gut microbiota accompanied by reduced body weight (49). In one animal study, DPP-4 inhibitor vildagliptin decreased *Oscillibacter* spp. and increased *Lactobacillus* spp (50). In mice treated with sitagliptin; one-third of the total species were occupied by *Ruminococcaceae*. These results suggested that sitagliptin might alter the gut microbiome to promote fermentation of complex plant-based carbohydrates and influence host metabolism (51). Other animal studies also showed that DPP-4 inhibitor increased the abundance of *Roseburia* and decreased *Blautia* with no effect on *Clostridium* (52).

In animal studies, GLP-1 receptor agonists reduced hyperglycaemia which was associated with a reduced abundance of *Romboutsia* and *Ruminiclostridium* as well as an increased abundance of *Prevotella* was associated with reduction of body weight (53). Neither GLP-1 receptor agonists nor DPP-4 inhibitors induced diversity of microbiome when used as an add-on therapy to metformin or sulphonylureas (SU) in the human study (54). Since metformin might have reshaped the microbiota, there might be little room for further changes by GLP-1 and DPP-4 inhibitors. However, in another clinical study involving 40 patients with T2D who were switched from metformin monotherapy to daily subcutaneous liraglutide injection for 4 months, there was an increase in the abundance of *Collinsella*, *Akkermansia*, and *Clostridium* genus (25).

Sodium–glucose-transporter-2 (SGLT2) inhibitors increase urinary glucose and sodium excretion resulting in a reduction in blood glucose, plasma insulin, blood pressure, and body weight (55). In the animal study, dapagliflozin, a SGLT2 inhibitor reduced the *Firmicutes* to *Bacteroidetes* ratio and increased the abundance of *Akkermansia muciniphila* (39). However, in a subsequent double-blind, randomized clinical trial comparing dapagliflozin and gliclazide, the latter being a sulphonylureas, in patients with T2D (26), neither drug induced any changes in the composition of gut microbiota. Sulphonylureas reduce blood glucose by directly stimulating insulin secretion. In a clinical trial, gliclazide, a sulphonylureas,

did not change the relative abundances of microbiota (23). In a 3-month observational study, another sulphonylureas, glipizide, also did not cause changes in the microbiota (23). In these clinical studies, the majority of patients were treated with metformin which was well known to alter the relative abundance of microbiota. Thus, the addition of dapagliflozin and gliclazide as add-on medications might not induce further significant effects. Besides, given their mechanisms of action which are largely independent of the gut, the neutral effects of SGLT2 inhibitors and sulphonylureas are not unexpected. However, most of these results in different WMs were not consistent due to the complex composition of the microbiota, the large variation between individuals in different cultures, and the differences in experimental design affecting by the effect of multi-therapy in treatment of WMs in human gut microbiota. Taken together, the conduct of well-designed, double-blind, placebo-controlled studies preferably in newly diagnosed, treatment-naïve patients with T2D and prediabetes are needed to clarify the mediating effects of microbiota on WM in influencing metabolic health.

## EFFECTS OF TCM ON THE GUT MICROBIOME IN T2D

Due to potential side effects of WM, notably hypoglycemia, as well as for reasons such as cultures, traditions, and social norms, TCM has always been an integral part of clinical practices and therapeutics in East Asian countries. Similar to metformin and acarbose, there is emerging evidence suggesting that TCM might alter the diversity of the gut microbiome with alteration of bile acid metabolism and increased production of SCFAs which contribute to the improvement of glucose metabolism. Herein, bile acids are cholesterol-derived metabolites that promote the intestinal absorption and transport of dietary lipids and play a key role in energy metabolism (56). **Table 2** summarise the effects of TCM on microbiota and metabolic effects.

Berberine is the main ingredient of TCM used for treating T2D. It is a natural plant alkaloid extracted from *Berberis aristata* and *Coptis chinensis* (Huanglian) (60). Berberine has reduced solubility in the gut and can permeate the gut wall. In a 12-week randomized clinical trial comparing berberine and placebo, berberine altered the gut microbiome composition with a 2-fold increase in *Bacteroides* spp. and *Proteobacteria* (61), a pattern similar to that due to metformin (11, 21). Berberine also induced cell death in harmful gut bacteria and enhanced the composition of beneficial bacteria including *Bifidobacterium adolescentis* and *Lactobacillus acidophilus* (61). Both berberine and metformin upregulated the AMPK pathway which reduced anabolism and promoted catabolism including glycolysis resulting in weight loss and reduced insulin resistance (62). Despite these beneficial effects, berberine depleted the SCFA-producing microbes including *Roseburia* spp., *Ruminococcus bromii, Faecalibacterium prausnitzii*, and *Bifidobacterium* spp. These two species (*Roseburia* spp. *and Bifidobacterium* spp.) are biomarkers indicative of a healthy gut microenvironment. Other researchers reported an inverse association of *Ruminococcus bromii* with bile acid metabolism with reduced formation of secondary bile acids by microbiota (57). In the intestine, bile acids undergo multistep biotransformation catalyzed by enzyme activities in gut bacteria, and the increase of *Ruminococcus bromii* suppresses bacterial 7α-dehydroxylase and leads to the reduction of secondary bile acids (63, 64). Although berberine lack some of the favourable effects of metformin on microbiota, it possessed beneficial effects exhibited by acarbose treatment.

Gegen Qinlian Decoction (GQD) is another popular TCM for the treatment of T2D. It comprises seven herbs including *Rhizoma coptidis*, *Radix scutellariae, Radix puerariae*, *Rhizoma anemarrhenae*, *Radix panacis uinquefolia*, *Radix paeoniae rubra* and *Rhizoma zingiberis* (65). The effects of GQD on microbiota were similar to that of berberine. GQD treatment altered the overall gut microbiota structure and enriched many butyrate-producing bacteria, including *Faecalibacterium*, *Bifidobacterium*, and *Gemmiger*. These changes in the gut milieu had been shown to attenuate intestinal inflammation and improve metabolic health including glucose metabolism. In the animal study, both berberine and GOD increased the plasma levels of SCFA with reduced fasting plasma insulin level (58). In another study, treatment with GQD enriched the abundance of Faecalibacterium prausnitzii which was negatively correlated

**TABLE 2** | Summary of the effects of traditional Chinese medicine on the composition of the gut microbiome in T2D patients.

| TCM | Year | Patients | Period | Study design | Microbes | Outcomes |
|---|---|---|---|---|---|---|
| Berberine | Zhang et al., 2020 (57) | 409 Patients with T2D treated with either berberine alone, probiotic+ berberine, probiotic alone or placebo. | 12 weeks | Randomized, double-blind, placebo-controlled trial | Berberine ↓ *Ruminococcus bromi* | Berberine reduced HbA1c, fasting and postprandial plasma glucose, fasting plasma triglyceride, total and low-density lipoprotein cholesterol |
| GQD | Xu et al., 2015 (58) | 187 patients with T2D treated with either GQD or placebo | 12 weeks | Randomized double-blinded placebo-controlled clinical trial | GQD ↑ *Faecalibacterium prausnitzii* | GQD reduced the mean fasting plasma glucose and HbA1c |
| AMC | Tong et al., 2018 (59) | 100 patients with T2D treated with either the metformin or AMC | 12 weeks | Randomized, open labelled randomized study RCT | AMC ↑ *Faecalibacterium* spp. | AMC reduced plasma glucose and lipids |

*GQD, Gegen Qinlian Decoction; AMC, specifically designed herbal formula (no full name provided).*

with fasting and 2-hour postprandial blood glucose and HbA1c as well as positively with insulin response as indicated by the HOMA-β index (66). One of the ingredients in GQD, *Radix scutellariae*, is commonly co-investigated with metformin (67). TNF-α was significantly reduced and the abundance of *Lactobacillus* and *Akkermansia* remarkably increased after metformin treatment with *Scutellaria baicalensis* when compared to metformin treatment with placebo.

JinQi Jiangtang (JQJT) is a formula used for the prevention of T2D. It contains *Rhizoma coptidis, Astragali Radix and Lonicerae Japnicae Flos.* In the animal study, treatment with JQJT tablets increased the abundance of species *Akkermansia* and reduced that of genus *Desulfovibrio*. Of note, reduced abundance of *Akkermansia spp* was correlated with inflammation in people with obesity (68). Other studies had reported that JQJT tablets modulated gut microbiota with increased formation SCFAs. The latter can provide energy and nutrition for the intestinal epithelium with improved gut health (69). There are limited studies on the effects of JQJT on microbiota in patients with T2D. In a 2-year multi-center randomized clinical trial involving 400 Chinese individuals with prediabetes, treatment with JQJT was associated with a lower incidence of diabetes compared to placebo with reduced blood glucose, triglyceride, albuminuria, and insulin resistance although there was no information on microbiota (70).

A modern herbal formula called AMC (no full name provided in the article) had been specifically developed for the treatment of T2D with hyperlipidemia. The herbs used in this formula included *Rhizoma Anemarrhenae, Momordica charantia, Coptis chinensis, Aloe vera*, and red yeast rice. In a randomized study comparing AMC and metformin in patients with T2D, AMC was similarly efficacious as metformin in reducing blood glucose and lipid levels. Both metformin and AMC enrich the abundance of beneficial bacteria *Blautia* spp., which correlated with improvements in glucose and lipid homeostasis. However, AMC showed better efficacy than metformin in improving HOMA-IR and plasma triglyceride *via* an increase of *Roseburia*, *Faecalibacterium, Gemmiger, Coprococcus*, and *un-Lachnospiraceae* (59).

## INTERACTION BETWEEN WM AND TCM

Current evidence suggested that both WM and TCM orchestrated different effects on the microbiome (**Figure 1**) to modulate glucose metabolism through different mechanisms. In East Asia with a large number of people with T2D, herbal medicines are frequently used as complementary therapies by patients treated with WM, notably metformin, although co-administration of TCM and WM is lacking in the clinical guideline. Few studies evaluated possible WM-TCM interactions including pharmacokinetics and pharmacodynamics. In recent human study, compared with administration of metformin and placebo, co-administration of metformin and berberine resulted in significant improvements in glycemic control, liver fat content, and body weight (71). In an animal study, compared with administration of metformin alone, co-administration of berberine and metformin resulted in changes in the gut microbiome due to reduced metformin degradation. These changes included an increased abundance of *Bacteroides fragilis*, *Clostridium perfringens*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter cloacae*, which might adversely affect the host immunity. These less desirable changes suggested berberine might attenuate the favourable effects of metformin on microbiota (72). Further investigations are warranted to evaluate the impacts of WM-TCM interactions on microbiota and human health.

## OTHER FACTORS AFFECTING DRUG-MICROBIOME INTERACTIONS

Host genomes, dietary habits, and physical activities are the most important factors that might confound drug-microbiome interactions. Within the same population, researchers reported considerable inter-individual as well as intra-individual variations in their microbiome patterns such as the ratio of *Firmicutes* to *Bacteroidetes* which are the two major phyla in the gut (73). These differences are most likely due to differences in dietary habits, physical activity, and consumption of different drugs such as antibiotics.

### Dietary Factors and Physical Activities

Dietary factor is directly interacting with gut microbiota and many research had indicated that different diets orchestrate the pattern of microbiota (74–76). Other researchers had reported that habitual dietary consumption caused changes in the composition of gut microbiota which in turn influenced the effects of their drug therapy. In Japanese patients with T2D taking acarbose, high rice intake was associated with the abundance of *Faecalibacterium* while high intake of potatoes was associated with a low abundance of health-promoting microbiota such as *Akkermansia* and *Subdoligranulum* (77). In another human study, compared to 20 obese women before metformin treatment, an increase of *Escherichia/Shigella* was found after 2 months of low-calorie diet and metformin treatment (78). This results did not suggest in other similar study design of human (22) and animal (79) studies when having metformin alone. These findings lent support to the hypothesis that diet-drug interaction may alter the microbiota to either attenuate or augment the therapeutic efficacy of WM or TCM.

In a human study, 26 subject sedentary lifestyle and prediabetes or T2D were increased exercise for 2 weeks, a decrease in the *Clostridium* genus was observed (8). In another study, 12-week intense exercise-induced changes in the gut microbiota in subjects with prediabetes with marked improvement in insulin resistance and reduced insulin level. This was accompanied by decreases in *Bacteroides xylanisolvens* and an increase in the abundance of *Streptococcus mitis* (80). However, no study investigated the effect of medicine and exercise on the gut microbiota in T2D.

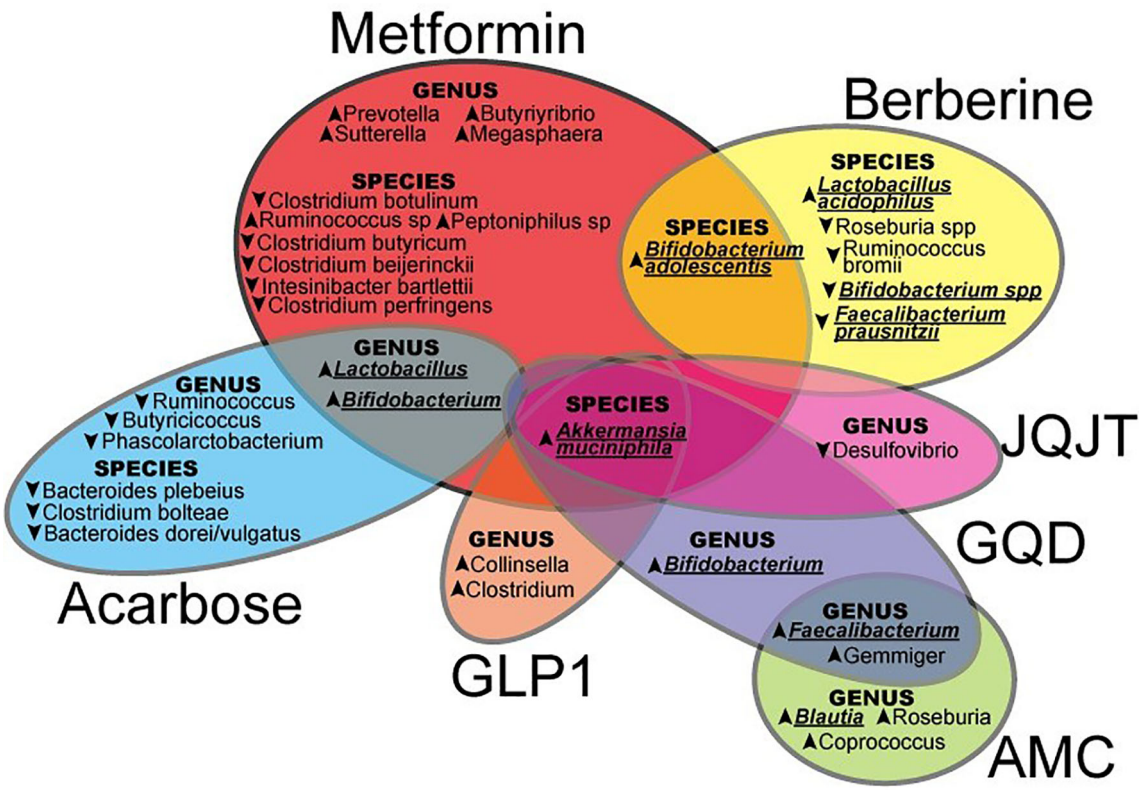


**FIGURE 1** | The effects of Western Medicine and Traditional Chinese Medicine in shaping the gut microbiota which may contribute to the control and prevention of type 2 diabetes (JQJT, JinQi Jiangtang; GQD, Gegen Qinlian Decoction; AMC, specifically designed herbal formula (no full name provided); GLP-1, Glucagon-like peptide-1.) In this review, summarized evidence suggested that both WM and TCM orchestrated different patterns on the microbiome, upward and downward arrows indicated an increase or decrease of certain microbiota by WM or TCM, and the particular microbiota underlined were possibly highlighted in the treatment of T2D.

## DRUG-GUT-MICROBIOTA CROSS-TALKS AND DRUG EFFICACY

Many oral glucose-lowering drugs might cause gastrointestinal side effects, partly due to fermentation of undigested carbohydrates by microbiota resulting in gas formation with altered transit time and gut permeability. These side effects might be alleviated using prebiotics or probiotics to improve treatment tolerance and glycemic control. Prebiotics and probiotics are microbiota-management tools for improving host health. Prebiotics are a group of nutrients in natural foods that are selectively utilized by host microorganisms conferring a health benefit and probiotics are health-related microbial strains and act as an oral supplement or added into food products (81). In a clinical study involving ten metformin-intolerant patients with T2D, administration of a readily dissolvable powder containing inulin, beta-glucan and polyphenols modulated the microbiome with improved metformin tolerance (82). Inulin and beta-glucan are metabolized in the colon by *Bacteroides* and *Prevotella* genera (83) with increased secretion of peptide YY and GLP-1. These changes were accompanied by reduced fasting plasma glucose and frequency of loose stool, a common side effect of metformin.

Since orally administered drugs may shape the gut microbiota, researchers suggested that probiotics might be used as an adjunctive to WM aimed at altering the diversity of microbiota with increasing SCFAs and enhanced glucose management. In a randomized placebo-controlled study, co-administration of probiotics (*Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., and *Saccharomyces* spp.) in 60 subjects with prediabetes or T2D, did not improve glycemic control but increased insulin sensitivity. There was an increase in the relative abundance of *Bifidobacterium breve* and *Akkermansia muciniphila* and *Clostridium XIVa*, albeit short of significance compared with the placebo group (84). Whether administration of prebiotics to augment the health-promoting effects of microbiota might be more effective than direct administration of health-promoting probiotics in improving drug tolerance or metabolic health is a subject that warrants further investigations.

### Future Perspectives

Much remains unknown on the effect of glucose-lowering WM and TCM on microbial composition and interaction with host factors. In addition to effects on blood glucose, changes in microbiota may also improve other cardiometabolic risk

factors. Modulation of the microbiota be part of a new therapeutic strategy against other diseases, such as non-alcoholic fatty liver disease (85), cardiovascular or even neurodegenerative disorders (86). For example, *Akkermanisa* spp., which is increased by metformin, was also highly correlated with weight loss (74). There is emerging evidence for a pro-inflammatory dysbiosis in neurodegenerative disorders such as Parkinson's disease. The decrease in anti-inflammatory genera such as *Blautia*, *Coprococcus*, *Roseburia*, and *Fecalibacterium (*87*)*, could potentially be reversed by metformin or acarbose. Finally, pharmacomicrobiomics should evaluate interactive effects between WM and TCM in the treatment of diabetes, where either beneficial or harmful drug interactions mediated *via* microbiota might occur.

## CONCLUSION

Type 2 diabetes is a disorder of energy metabolism due to complex interplays amongst the ecosystem, host, and microbiome. The natural history of obesity, prediabetes and diabetes are associated with inter-individual and intra-individual diversity of microbiota. Diabetes-associated dysbiosis is characterized by a reduction in gram-positive members of the beneficial microbiota such as *Blautia*, *Rumminococcaceae*, and gram-negative *Akkermanisa* species with reduced production of SCFA and dysregulation of bile acid metabolism which can adversely affect metabolic health.

Glucose lowering drugs can alter glucose, lipid, and fat metabolism and modulate inflammatory responses by re-shaping the composition of the microbiome which in turn can affect immune cells directly and indirectly through metabolites such as lipopolysaccharide and SCFAs, alteration of gut permeability, and whole gut transit time. Host-gut microbiota interaction is central in bile acid metabolism and cell signalling and can be modulated by medications. The effects of these changes in gut microbiota might contribute to the diversity in disease phenotypes including hormones and inflammatory cytokines. Both WM (e.g. metformin and acarbose) and TCM (berberine based) have been shown to improve the abundance of beneficial bacteria, such as *Blautia* spp., *Akkermanisa* spp., *and Faecalibacterium*, and reduce the production of secondary bile acids which might contribute towards their metabolic effects including their side effects. Integration of WM and TCM may promote different health-related microbiota and suppress the pathogenic microbiota, such as *Desulfovibrio*. Given the expanding knowledge in the field of microbiome and the availability of high throughput sequencing, further investigations on the modulating effects of microbiota on the efficacy and side effects of WM and TCM will provide novel insights and open a new avenue for reducing the burden of T2D and non-communicable diseases.

## AUTHOR CONTRIBUTIONS

NC and EC conceived of the presented idea. NC drafted the manuscript and designed the figures. JC and EC devised the main conceptual ideas and proof outline. JC encouraged NC to investigate the Chinese Medicine and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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# Is Intestinal Dysbiosis-Associated With Immunosuppressive Therapy a Key Factor in the Pathophysiology of Post-Transplant Diabetes Mellitus?

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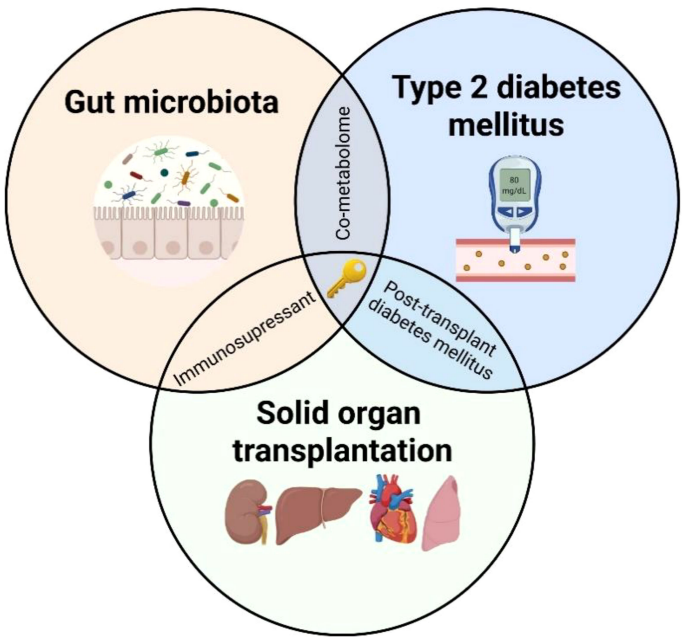
Post-transplant diabetes mellitus (PTDM) is one of the most common and deleterious comorbidities after solid organ transplantation (SOT). Its incidence varies depending on the organs transplanted and can affect up to 40% of patients. Current research indicates that PTDM shares several common features with type 2 diabetes mellitus (T2DM) in non-transplant populations. However, the pathophysiology of PTDM is still poorly characterized. Therefore, ways should be sought to improve its diagnosis and therapeutic management. A clear correlation has been made between PTDM and the use of immunosuppressants. Moreover, immunosuppressants are known to induce gut microbiota alterations, also called intestinal dysbiosis. Whereas the role of intestinal dysbiosis in the development of T2DM has been well documented, little is known about its impacts on PTDM. Functional alterations associated with intestinal dysbiosis, especially defects in pathways generating physiologically active bacterial metabolites (e.g., short-chain fatty acids, trimethylamine N-oxide, indole and kynurenine) are known to favour several metabolic disorders. This publication aims at discussing the potential role of intestinal dysbiosis and dysregulation of bacterial metabolites associated with immunosuppressive therapy in the occurrence of PTDM.

**Keywords: post-transplant diabetes mellitus, type 2 diabetes mellitus, immunosuppressant, intestinal dysbiosis, bacterial metabolites**




**GRAPHICAL ABSTRACT** |

## 1 INTRODUCTION

Solid organ transplantation (SOT) is the best replacement therapy in numerous cases of organ failure or end-stage organ dysfunction (*e.g.*, kidney, liver, heart, or lung). Immune tolerance of the transplanted organ requires a complex and life-long immunosuppressive therapy, involving combinations of drugs from six main classes: 1) anti-proliferative agents (azathioprine, mycophenolic acid); 2) calcineurin inhibitors (cyclosporine, tacrolimus); 3) mammalian target of rapamycin (mTOR) inhibitors (sirolimus, everolimus); 4) co-stimulation blockers targeting CD80/CD86 (belatacept); 5) anti-lymphocyte polyclonal or monoclonal antibodies (*e.g.*, anti-thymocyte globulins, basiliximab); and 6) corticosteroids (*e.g.*, prednisolone) (1, 2). The immunosuppressive strategy along time consists of: an induction phase that involves anti-lymphocyte antibodies, corticosteroids and the use of higher doses of "maintenance" immunosuppressants such as antimetabolites and calcineurin inhibitors; a life-long maintenance phase with different combinations of classes 1 to 4 with or without corticosteroids (3); and treatment of rejection, using boluses of corticosteroids, anti-thymocyte globulins, increased doses of maintenance drugs, and potentially other drugs in case of antibody-mediated rejection (ABMR) (4). Unfortunately, these therapeutic regimens increase the risk of opportunistic bacterial, viral, and fungal infections (5) and expose patients to numerous adverse effects and several metabolic disorders.

Post-transplant diabetes mellitus (PTDM) is a common and deleterious co-morbidity, which significantly contributes to adverse outcome. PTDM is an endocrine and metabolic disease characterized by a dysfunction of pancreatic β-cell, insulin resistance, and high blood glucose. Among the risk factors of PTDM, several are common with type 2 diabetes mellitus (T2DM) (*e.g.*, age, abdominal obesity) whereas others are transplant-specific (*e.g.*, immunosuppressive drugs with diabetogenic properties, infection, and post-transplant weight gain) (6, 7). Immunosuppressive drugs can influence gut homeostasis through an impact on intestinal epithelial cells or organs associated with the digestive tract and induce changes in the richness and diversity of the gut microbiota. This drug-microbiota relationship may directly or indirectly affect the anti-rejection treatment efficacy as well as disrupt the microbiota balance and favour the development of metabolic disorders (8).

Although preventative and therapeutic strategies are being deployed to prevent PTDM, its incidence remains high. To improve the effectiveness of such strategies, it is necessary to better understand PTDM pathophysiology. Our hypothesis is that modifications of the gut microbiome, also named intestinal dysbiosis, a well-known contributor to type 2 diabetes mellitus (T2DM) in the non-transplant population, play an even larger role in the pathogenesis of PTDM. To substantiate this hypothesis, we herein provide a picture of the impact of SOT and immunosuppressive therapy on the gut homeostasis including gut microbiota. Subsequently, we discuss the potential role of intestinal dysbiosis in the development of PTDM based on knowledge gained from T2DM and provide arguments in favour of monitoring the microbiota diversity and function to decipher PTDM pathophysiology.

# 2 CURRENT KNOWLEDGE ON POST-TRANSPLANT DIABETES MELLITUS

## 2.1 Diagnosis and Incidence

PTDM is a one of the most important comorbidities associated with SOT. The evaluation of its incidence among transplant patients has suffered from the lack of a consensual definition. The first international consensus guidelines about new-onset diabetes after transplantation (NODAT) were published in 2003 (9). A second international consensus conference was held in 2013 to review the criteria and available evidence and proposed an update to the previous guidance (10). Among many recommendations, the first was to enlarge the notion of NODAT to that of PTDM. PTDM encompasses several complex clinical entities and includes hyperglycemia in the post-transplant period resulting from known or unknown pre-existing diabetes, insulin resistance or insulinopenia, transient hyperglycemia, and NODAT. Therefore, this definition encompasses pre-transplant in addition to "new-onset" diabetes (10). Currently, the different diagnostic criteria for PTDM (**Table S1**) are based on those of the American Diabetes Association and on the World Health Organization criteria for non-transplant patients (11).

The incidence of PTDM ranges from 10 to 40% depending on the transplanted organ. Recently, PTDM has been reported to occur in 10-20% of kidney, 20-40% of liver or lung, and 20-30% of heart transplant recipients (6). These large ranges may be explained by the type of organ, the presence of modifiable and non-modifiable risk factors, and the follow-up duration (5). The development of diabetes in transplant recipients is associated with a higher risk of graft failure, patient death, and other adverse outcomes (*e.g.*, cardiovascular disease and infection) (12). More specifically, PTDM is associated with a higher incidence of cardiovascular disease for liver and kidney transplant recipients (13, 14). Heart transplant recipients with PTDM present an increased risk of comorbidities and premature death (15). Moreover, PTDM in lung transplant recipients is associated with shorter survival (16).

## 2.2 Pathogenesis and Risk Factors

PTDM shares common features with type 2 diabetes mellitus (T2DM) such as insulin resistance, hypertriglyceridaemia, cardiovascular events, and chronic low-grade inflammation. Hyperglycaemia in PTDM is associated with pancreatic β-cells dysfunction and decreased insulin sensitivity (6, 17). Studies in PTDM patients reported impaired insulin-mediated glucose uptake in peripheral tissue, impaired insulin-mediated suppression of hepatic glucose output (18) and insufficient incretin release leading to an increase of glucagon release by the pancreas (19). Pre-existing risk factors common to PTDM and T2DM, such as age, abdominal obesity, family history, and ethnicity favour the development of PTDM. The morphotype in the pre-transplant period could predict to some extent the development of PTDM in kidney transplant recipients (20). Moreover, several T2DM-associated single nucleotide polymorphisms (SNPs) in interleukin genes (*e.g.*, IL-7R, IL-2, and IL-17R) are associated with increased pro-inflammatory pathways and PTDM development (21, 22).

Among transplant-related risks factors, numerous studies have demonstrated the involvement of certain immunosuppressive drugs in the development of NODAT. Calcineurin inhibitors dysregulate the function and growth of pancreatic β-cells through the calcineurin/NFAT signalling pathway. Corticosteroids are known to decrease the secretion of, and sensitivity to, insulin (23). Sirolimus favours insulin resistance and decreases pancreatic β-cell proliferation too (24). Consistently, mTOR inhibitors are associated with a higher risk of PTDM (25). Above all, recent analysis pointed toward the contribution of immunosuppressants to the dysregulation of genes involved in insulin production and secretion (24). Viral infections are a source of inflammation and represent yet another risk factor of PTDM. Numerous studies have reported an increased risk of PTDM in kidney and liver transplant recipients positive for the hepatitis C virus (HCV) (26–28) or in kidney transplant recipients positive for the cytomegalovirus (CMV) (29). Although associations between these viral infections and PTDM are generally attributed to the promotion of a pro-inflammatory environment as well as to pancreatic β-cell dysfunctions, extensive studies are missing.

Actually, the preventative strategies against PTDM involve lifestyle (e.g., dietary, physical activity) modifications or adapted immunosuppressive regimens (30). However, the frequency of PTDM has not decreased significantly over the last decade, suggesting that current knowledge is not sufficient and that uncharacterized phenomena contribute to PTDM. Several risk factors presented above (*e.g.*, obesity, immunosuppression, infection) are accompanied by an imbalance in the diversity of the gut microbiome, called intestinal dysbiosis, metabolic disorders and increased intestinal permeability. These alterations are well known to favour T2DM in the non-transplant population (31). Therefore, the drastic dynamic changes of the gut microbiota during SOT may contribute even more to the pathogenesis of PTDM.

# 3 ALTERATIONS OF GUT HOMEOSTASIS IN SOLID ORGAN TRANSPLANTATION

Gut homeostasis is highly dependent on the intimate crosstalk between the gastrointestinal tract and the gut microbiota. The gut microbiota represents the populations of commensal microorganisms that reside in the gastrointestinal tract and participate in the intestinal barrier integrity. Recent scientific advances have underlined the fundamental role of this microbiota in the regulation of the immune system, as well as the close relationship between intestinal dysbiosis and the occurrence of numerous local or systemic diseases (mainly cardiovascular or metabolic disorders) (32, 33). The gut microbiota may therefore represent an actionable target to improve immune tolerance and long-term graft survival. The diversity, richness, and activity of its resident microorganisms are constantly being modified under the influence of various factors (*e.g.*, genetic, dietary, environmental, and therapeutic). It is worth mentioning that in transplantation, the nature of the transplanted organ, the various pre- and post-transplant

pathologies, and multiple therapies accentuate the gut microbiota variability. The dynamic changes of the gut microbiota have been widely studied in some diseases, but very seldom in transplant patients. We summarize below current knowledge about the relationships between the gut microbiota and SOT outcomes (**Figure 1A**), including the impact of the immunosuppressive protocol and the occurrence of post-transplant co-morbidities.

## 3.1 Dynamic Changes of the Gut Microbiota in Transplant Patients

The surgical procedure of transplantation which is generally an abdominal act, represents a high risk of intestinal dysbiosis (34, 35). Regarding the post-transplantation period, a cohort study in kidney transplant recipients reported changes in gut microbial diversity in favour of an increase in Proteobacteria, a phylum that includes potentially virulent pathogens (*e.g., Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) (36). The faecal microbial diversity was decreased in some patients with post-transplant complications (diarrhoea, acute rejection, urinary tract infection) (37). In addition, the diversity of the gut microbiota was significantly lower and the levels of Proteobacteria higher with abundant *Escherichia coli* in kidney transplant recipients compared to healthy control (38). In liver transplant recipients, this diversity transiently decreased two weeks after transplantation and then gradually increased back to reach the pre-transplantation levels after 5 weeks (39). Anti-infectious agents used in transplanted patients to prevent opportunistic infections, mainly antibiotics, are known to affect the gut microbiota homeostasis and to promote intestinal dysbiosis (36, 40).

## 3.2 Effect of the Immunosuppressive Therapy on the Gut Homeostasis

### 3.2.1 Interactions Between the Immunosuppressive Drugs and the Gut Microbiota

A recent review from Gabarre *et al.* has provided a thorough overview of the bidirectional interaction between the immunosuppressants and the gut microbiota (8). The use of the anti-proliferative agent, mycophenolic acid, initially known for its antibacterial, antifungal and antiviral properties, is associated to a

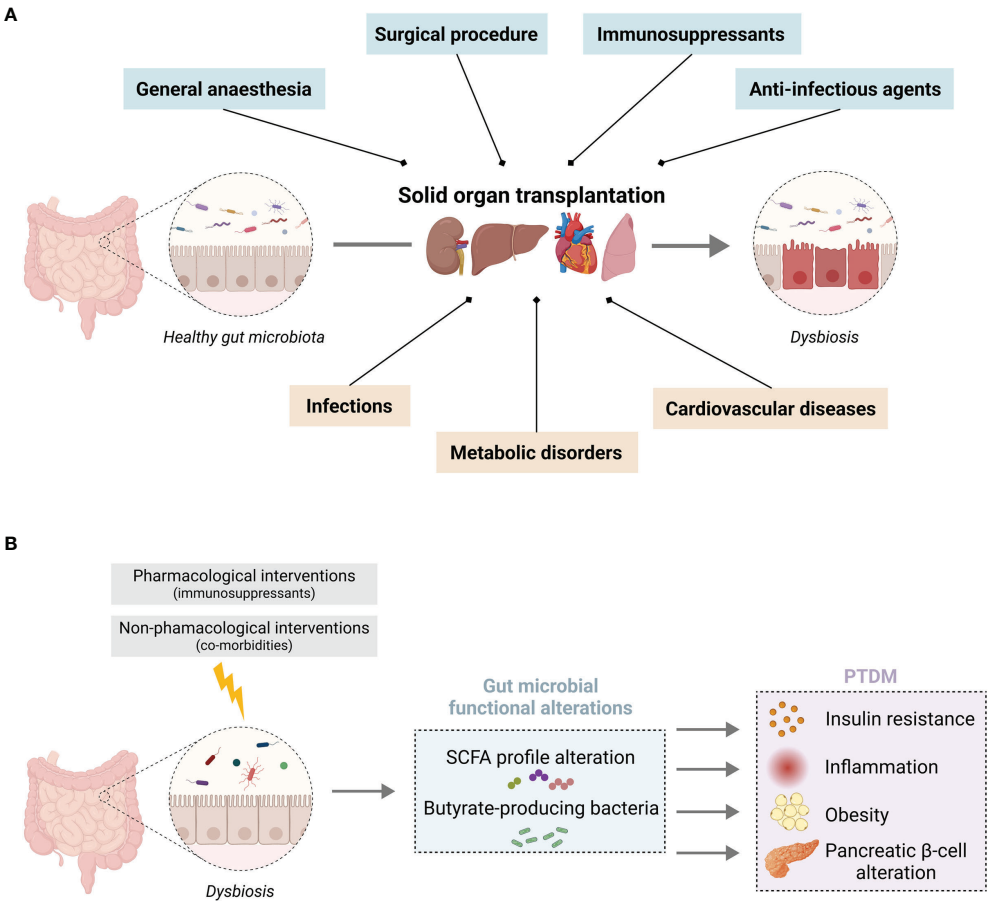



**FIGURE 1** | Solid organ transplantation associated pharmacological and non-pharmacological interventions on gut microbiota homeostasis **(A)** Overview of the impact of SOT on gut microbiota homeostasis associated with (blue) the surgical procedure, anti-infectious prophylaxis, immunosuppressants, and (orange) SOT-related co-morbidities. **(B)** Putative consequences of an imbalance in SCFA-producing bacteria induced by SOT, which favour the development of PTDM. Illustration created with BioRender.com.

decrease in the diversity of the gut microbiota in kidney transplant recipients (38, 41). Further investigations in preclinical models have revealed an alteration of the intestinal microbiota in mycophenolic acid-treated mice with an expansion of bacteria belonging to the Proteobacteria phylum (42, 43). In addition, a decrease of bacterial metabolites was also observed in a mouse model of mycophenolate-induced enteropathy (44). Mycophenolic acid is thought to selectively promote the expansion of β-glucuronidase-expressing bacteria of Enterobacteriaceae family (42, 43). The bacterial β-glucuronidase activity promotes enterohepatic recirculation of mycophenolic acid and increases its exposure to intestinal epithelial cells that could probably explain the occurrence of gastrointestinal adverse effects. Regarding corticosteroids, rats treated orally with dexamethasone sodium phosphate showed a decrease in the richness and diversity of their gut microbiota (45). Prednisolone-treated mice showed a reduction in the population of Bacteroidetes and an increase in Firmicutes in faecal samples (46). For calcineurin inhibitors, a study reported altered microbiota in high-dose tacrolimus-treated mice (8, 47). Another study based on intraperitoneal injection of tacrolimus in rats showed that the relative abundance of several bacterial species in the faeces was decreased (48). The composition of the gut microbiota can affect the metabolism of tacrolimus as some commensal gut bacteria (*e.g., Faecalibacterium prausnitzii*) have been shown to convert it to less potent metabolites (49).

These studies showed in the one hand, that several immunosuppressive drugs induced intestinal dysbiosis leading to change in microbial diversity favouring the increase of opportunistic pathobionts and in other hand, that the gut microbiota influenced the immunosuppressive drugs metabolism and efficacy. However, the characteristics of gut microbiota changes differ across drugs and studies and systematic and longitudinal investigations that could provide insight into these trends are still lacking. However, the above-mentioned review lists the immunosuppressants inducing intestinal dysbiosis and provides an overview of the related changes in the microbiota (8).

### 3.2.2 Immunosuppressive Drugs Impacts on Intestinal Barrier Integrity and Pancreas Homeostasis

In transplant patients, immunosuppressive drugs can alter the intestinal barrier integrity and favour intestinal permeability. Intestinal permeability is characterized by a loss of the gut epithelial wall integrity allowing different sizes of compounds to enter the systemic circulation (food antigens, commensal or pathogens bacteria, and their metabolites) (50). By using an intestinal epithelial cell line, Qasim *et al.* have demonstrated the potential of mycophenolic acid to alter tight junction proteins expression and distribution and induce intestinal permeability that may be responsible for gastrointestinal adverse effects observed in transplant patients (51). This intestinal permeability could also have deleterious consequences such as chronic systemic inflammation (52). Another study has demonstrated that tacrolimus and sirolimus by inhibiting cell viability and inducing reactive oxygen species formation, can promote major changes in intestinal barrier function (53).

The immunosuppressive drugs can also alter the homeostasis of organs associated with the digestive tract. We herein only focus on the influence of the pancreas homeostasis as it plays a key role in the regulation of nutrient digestion by releasing digestive enzymes and glucose homeostasis (54). Some rare cases of drug-induced pancreatitis have been reported under tacrolimus (55) or mycophenolic acid (56) treatments. However, there is no strong evidence of the direct impact of the immunosuppressive drug on the pancreas homeostasis that could rationalize the occurrence of PTDM. Therefore, the whole impact of the environment (*i.e.*, dysbiosis, immunosuppressive therapy, co-morbidities) could account for the development of PTDM.

## 3.3 Impact of SOT-Related Co-Morbidities on the Gut Microbiota

In the first months post-transplantation, patients are at high risk of developing infections due to a weakened immune system. Serious infections can be caused by commensal or nosocomial bacterial (*e.g.*, *Pseudomonas*, *Klebsiella*, *Escherichia*), viral (*e.g.*, cytomegalovirus, Epstein-Barr virus, influenza) or fungal (*e.g.*, *Candida* or *Aspergillus* species) pathogens (57). *Clostridium difficile*, a frequent perpetrator of nosocomial infection (7.4% prevalence in SOT patients), is linked with the emergence of intestinal dysbiosis (58, 59). Gut microbiome alteration is frequently associated with these infections and is characterized by an enrichment of opportunistic pathogens and a depletion of beneficial commensals (60, 61). For example, a preclinical study has reported variations in gut microbiota diversity in cytomegalovirus-infected mice (62).

The main metabolic complications after SOT include PTDM, non-alcoholic fatty liver disease, dyslipidaemia, and obesity. These metabolic disorders may increase the risk of cardiovascular events (hypertension, coronary artery disease, stroke, arteritis) and affect post-transplant graft outcomes (63–66). For instance, non-alcoholic fatty liver disease is associated with an increase in Proteobacteria, leading to gut dysbiosis (67, 68). Immunosuppressive drugs such as corticosteroids and calcineurin inhibitors can favour hypertension and weight gain (69). This weight gain of SOT patients is critical in post-transplant period, since obesity has been significantly associated with a higher overall mortality and reduced allograft survival particularly in renal transplant patients (70). These metabolic complications can have a deleterious effect on gut microbiota homeostasis. Obesity affect the diversity of intestinal microbiota, with an increase in Firmicutes and a reduction of Bacteroidetes in a mice model (71). Intestinal dysbiosis has been observed in obese people, with an increased Firmicutes-to-Bacteroidetes ratio (72).

In summary, SOT therapy is accompanied by intestinal dysbiosis arising from a combination of factors including lifestyle and dietary changes, surgical procedure, and pharmacological treatments (*e.g.*, anti-infectious prophylaxis, immunosuppressant). Regarding the gut microbiota-diabetes relationship, several studies have demonstrated a huge diversity imbalance in diabetes patients (31). Given the predominant role of this dysbiosis in the pathogenicity of T2DM, the hypothesis of its involvement in PTDM seems strong.

## 4 POTENTIAL INVOLVEMENT OF INTESTINAL DYSBIOSIS TO THE PATHOPHYSIOLOGY OF PTDM

In this section, we will put an accent on the gut microbiome changes observed in transplant recipients that are common with non-transplant T2DM. In this context, we will describe the putative impact of intestinal dysbiosis on the bacterial metabolites and more precisely on short-chain fatty acids (SCFA) and their possible role in the development of PTDM (**Figure 1B**).

As previously mentioned, several immunosuppressive drugs induce intestinal dysbiosis, generally characterised by a reduction in the phylum of Bacteroidetes, contrasting with an expansion of the phylum of Firmicutes (73). The same tendency has been observed in T2DM patients (74). This increased Firmicutes-to-Bacteroidetes ratio was associated with an impairment of nutrient absorption and glucose tolerance, which pave the way for T2DM (73). Moreover, the relative abundance of Proteobacteria is increased in kidney and liver transplant recipients (36, 38), similarly to T2DM patients (74, 75). Some bacterial strains belonging to this phylum are known to favour pathogenic infections (*e.g.*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) (36, 38). Gut microbiome changes can induce global metabolic disorders. Indeed, the gut bacterial ecosystem ensures the production of microbial metabolites (*e.g.*, SCFA, trimethylamine N-oxide, indole and kynurenine). These metabolites constitute the communication system of the host-microbiome crosstalk (76). Among them, SCFA are the most commonly studied small metabolites produced by the gut microbiota and they represent a robust link between the microbiota and systemic inflammatory diseases, as demonstrated by recent studies (33, 75).

SCFA, and more precisely acetate, propionate, and butyrate, come from the fermentation of indigestible carbohydrates. SCFA are pharmacologically active and can exert their numeral pharmacological functions by either stimulating G-protein-coupled receptors (GPCR41/43/109A) or can be absorbed by colonocytes through multiple monocarboxylate transporters (*e.g.*, sodium-coupled monocarboxylate transporters (SMCT1), monocarboxylate transporter (MCT1/4/5) (77). In the systemic circulation, they can participate in the regulation of several organs (*e.g.*, liver, lung, brain) (33, 78, 79). For example, they can decrease allergic inflammation in the lungs, or can be used as a source of energy by the kidneys, the myocardium and other muscles (80). SCFA facilitate IL-10 synthesis through the polarization of T-cells towards regulatory T-cells, which exhibits anti-inflammatory properties (81). They also exert a positive effect on intestinal cell homeostasis through the maintenance of the epithelial barrier function through the expression of tight junctions that decrease intestinal permeability (82).

Alteration of SCFA profiles has been observed in T2DM patients, with a significant reduction of faecal propionate and butyrate concentrations as compared to control subjects (83). Moreover, a metagenome-wide association study showed a decrease in the abundance of some universal butyrate-producing bacteria in T2DM patients such as those observed in transplant recipients (8, 47, 84). A European cohort study reported the decrease of butyrate-producing bacteria (such as *Roseburia* species and *Faecalibacterium prauznitzii*) in the gut microbiota of women with T2DM (85). These studies provide evidence that T2DM and SOT have in common SCFA-producing taxa alterations leading to decreased SCFA production. Butyrate and propionate influence glucose metabolism through the activation of intestinal gluconeogenesis, while acetate and propionate are substrates for gluconeogenesis and lipogenesis in the liver (86). SCFA play a role in blood glucose concentration by favouring the secretion of incretin hormones, as demonstrated by the butyrate-induced secretion of glucagon-like peptide 1 (GLP1) in a pre-clinical model (87). At the cellular level, the binding of SCFA to GPR41 and GPR43 in the enteroendocrine L-cells leads to increased GLP1 and peptide YY levels, which improve cell sensitivity to insulin and promote satiety. Furthermore, SCFA play a protective role against obesity and insulin resistance (73, 88) and have anti-inflammatory properties, especially butyrate. A decrease of butyrate-producing bacteria may favour metabolic inflammation, which in turn clearly induces insulin resistance and foster T2DM development (75). At the opposite, incubation of neutrophils with SCFA *in vitro* suppressed pro-inflammatory makers increased in T2DM, such as IL-6 and TNF-α (89). A recent experimental study showed that butyrate and acetate protected pancreatic β-cells against stressful conditions and alleviated metabolic stressor-induced apoptosis, mitochondrial dysfunction and ROS overproduction (88). Moreover, by stimulating their receptors, SCFA have been involved in the regulation of pancreatic β-cells function and insulin secretion (90). Overall, these studies demonstrated the important role of SCFA in the pathophysiology of diabetes through various mechanisms of action, which have been well detailed in a recent review (89). The decreased richness of SCFA-producing bacteria in SOT may therefore promote and/or contribute to the development of PTDM.

## 5 CONCLUSION

This article provides hints in favour of a possible association between intestinal dysbiosis and PTDM, based on complementary and coherent scientific evidence. Further investigations are required to reinforce the descriptive data available for SOT. Characterising gut microbiota composition would help to understand the mechanisms and/or to identify predictive biomarkers of PTDM. The measurement of SCFA concentrations in blood and/or faeces as indicators of the gut microbiota functionality in the pre- and post-transplant periods could also make the case stronger. Moreover, dietary supplementation with SCFA as a postbiotic could restore the gut microbiota homeostasis and constitute a complementary therapy for glucose lowering in PTDM. A recent paper stressed that the implementation of an effective PTDM prevention strategy requires relevant identification of at-risk patients, solid knowledge of its pathogenesis and early detection tools (30). Monitoring the gut microbiota in SOT would comply with these objectives since it could help decipher the pathophysiology PTDM and detect patients at increased risk early.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

QF, MJ and CB drafted the paper with the input of all authors. This work has been done under the supervision of NP, PM and RL. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.898878/full#supplementary-material

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# Effects of *PPARD* gene variants on the therapeutic responses to exenatide in chinese patients with type 2 diabetes mellitus

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**Background:** Exenatide is a GLP-1R agonist that often exhibits considerable interindividual variability in therapeutic efficacy. However, there is no evidence about the impact of genetic variants in the *PPARD* on the therapeutic efficacy of exenatide. This research was aimed to explore the influence of *PPARD* gene polymorphism on the therapeutic effect of exenatide, and to identify the potential mechanism futher.

**Methods:** A total of 300 patients with T2DM and 200 control subjects were enrolled to identify *PPARD* rs2016520 and rs3777744 genotypes. A prospective clinical study was used to collect clinical indicators and peripheral blood of T2DM patients treated with exenatide monotherapy for 6 months. The SNaPshot method was used to identify *PPARD* rs2016520 and rs3777744 genotypes, and then we performed correlation analysis between *PPARD* gene variants and the efficacy of exenatide, and conducted multiple linear regression analysis of factors affecting the therapeutic effect of exenatide. HepG2 cells were incubated with exenatide in the absence or presence of a PPARδ agonist or the siPPARδ plasmid, after which the levels of GLP-1R and the ratio of glucose uptake were determined.

**Results:** After 6 months exenatide monotherapy, we observed that homeostasis model assessment for insulin resistance (HOMA-IR) levels of the subjects with at least one C allele of the *PPARD* rs2016520 were significantly lower than those with the TT genotype, which suggested that the *PPARD* rs2016520 TT genotype conferred the poor exenatide response through a reduction of insulin resistance, as measured by HOMA-IR. The carriers of G alleles at rs3777744 exhibited higher levels of in waist to hip ratio (WHR), fasting plasma glucose (FPG), hemoglobin A1c (HbA1c) and HOMA-IR compared to individuals with the AA genotype following 6 months of exenatide treatment, potentially accounting for the lower failure rate of exenatide therapy among the AA homozygotes. In an insulin resistant HepG2 cell model, the PPARδ

agonists enhanced exenatide efficacy on insulin resistance, with the expression of GLP-1R being up-regulated markedly.

**Conclusion:** These data suggest that the *PPARD* rs2016520 and rs3777744 polymorphisms are associated with exenatide monotherapy efficacy, due to the pivotal role of PPARδ in regulating insulin resistance through affecting the expression of GLP-1R. This study was registered in the Chinese Clinical Trial Register (No. ChiCTR-CCC13003536).

KEYWORDS

***PPARD* gene, genetic variant, type 2 diabetes mellitus, exenatide, insulin resistance**

## Introduction

Within the past few decades, the prevalence of type 2 diabetes mellitus (T2DM) has risen at an astounding rate over the world (1). T2DM is a metabolic disease caused by a complex combination of environmental and genetic factors, characterized by impaired insulin secretion and insulin resistance (2). Genome-wide association studies (GWAS) have led to the identification of hundreds of risk genes, including peroxisome proliferator-activated receptor δ gene (*PPARD*), associated with T2DM susceptibility or abnormal indicators of metabolism (3). *PPARD* is located on chromosome 6p21.1-p21.2, and its coding product PPAR-δ (also named PPAR-β) is a member of the peroxisome proliferator activated receptor family, which is widely distributed in the liver, kidney, cardiac and skeletal muscle, adipose tissue, brain, pancreatic and vasculature (4). *PPARD* was not observed as the susceptibility gene for T2DM in a case-control clinical study conducted in a Korean population in 2004, but *PPARD* variants were founded to be associated with elevated fasting plasma glucose (FPG) and body mass index (BMI) (5). Studies in Chinese Han population have shown that *PPARD* rs2016520 variant (also named +294T > C or -87T > C) is associated with blood glucose, insulin level and insulin resistance, and is a key factor affecting the development of metabolic syndrome and T2DM (6, 7). Studies in Mexican population have produced similar results (8). Meanwhile, pathogenesis research already pointed that PPAR-δ plays an important role in insulin resistance and islet β-cell function (9–11). More recently, PPAR-δ activation came into focus as an interesting novel approach for the treatment of metabolic syndrome. Both preclinical and clinical studies showed that PPAR-δ specific agonist therapy enhanced β-oxidation, decreased free fatty acid, and improved insulin sensitivity (12, 13). Current studies have demonstrated the effect of *PPARD* on metabolic metrics, but the mechanisms responsible for this effect are not well characterized.

Exenatide is a glucagon-like peptide 1 (GLP1) analogue that exerts its pharmacological effects through activating glucagon-like peptide 1 receptor (GLP1-R). It mirrors many of the effects of GLP-1 and improve glycemic control through a combination of mechanisms, which includes glucose-dependent stimulation of insulin secretion, suppression of glucagon secretion, slowing of gastric emptying and reduction of appetite (14, 15). The results of a multi-center randomized controlled clinical trial showed that there were significant individual differences in glycemic control, islet function, and body mass index in T2DM patients undergoing exenatide monotherapy for 48 weeks (16). Our research team conducted a previous retrospective clinical study to investigate the hypoglycemic efficacy of 148 T2DM patients treated with exenatide and discovered that the proportion of T2DM patients who did not respond to exenatide treatment was as high as 37.84% (17). The non-response of exenatide treatment not only affects the glycemic standard of T2DM patients and reduces the medication compliance, but also brings a heavy economic burden to patients. Some studies have shown that gene variants can affect the stimulatory effect of GLP-1 receptor agonists on insulin secretion (18–20). The TCF7L2 rs7903146 mutation was found to attenuate GLP-1-induced insulin secretion in German and Danish populations (18, 19). Another study suggested that mutations in the WFS1 gene attenuated GLP-1-induced insulin secretion, but not in relation with insulin sensitivity (20). Therefore, genetic factors are essential to the efficacy of exenatide. We analyzed the GLP-1R promoter using the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) and JASPAR database (http://jaspar.genereg.net/), and found the potential PPARδ binding site (Supplementary Figure S1).

Pharmacogenomic studies have identified genetic factors as an important cause of individual differences in the efficacy of hypoglycemic drugs (2, 21, 22). However, it remains unknown whether *PPARD* single nucleotide polymorphisms (SNPs) have

the same influence on the therapeutic effects of exenatide. In this study, we investigated the association between *PPARD* variants and the efficacy of exenatide in newly diagnosed Chinese T2DM patients who received exenatide monotherapy for 6 months, and further explored the potential mechanism.

# Materials and methods

## Participants and study design

A total of 300 patients with T2DM (196 male and 104 female) and 200 healthy controls (139 male and 61 female) were enrolled for *PPARD* variants analysis. T2DM patients and healthy subjects were recruited from the Department of Endocrinology and Health Screening Center, Affiliated Hospital of Xuzhou Medical University, Xuzhou, China. T2DM was diagnosed according to the 1999 World Health Organization criteria. The inclusion criteria were: newly diagnosed T2DM without drug therapy; 25 to 70 years old; Hemoglobin A1c (HbA1c) 7%-12%; BMI 20-35 kg/m$^2$; Stable body weight (≤10% change within 3 months); Female subjects were required to use birth control pills during the three months prior to screening and during the study, or surgical contraception, or postmenopausal women. Subjects with acute or severe chronic diabetic complications, serious comorbid diseases, New York Heart Function Scale (NYHA) III~IV, severe osteoporosis or a history of fractures, alanine aminotransferase (ALT) and aspartate transaminase (AST) ≥2.5 times of upper limit, or serum creatinine level ≥133 μmol/L, severe gastrointestinal dysfunction, ongoing use of weight-loss drugs, glucocorticoids, drugs that affecting gastrointestinal motility, transplant therapy drugs, any investigational drugs, a history of pancreatitis, or serum triglyceride level ≥5 mmol/L were excluded. A total of 105 newly diagnosed T2DM patients (73 male and 32 female) with different *PPARD* rs2016520 and rs3777744 genotypes were randomly selected to receive exenatide injection subcutaneously for 6 months and completed the follow-up. During the 1st week to the 4th week, exenatide was given 5 μg once, twice a day, after which the dose was adjusted to 10 μg once, twice a day. The protocol was approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University. Written informed consent was obtained from each participant before taking part in the study.

## Anthropometric and biochemical measurements

The general anthropometric parameters such as height, weight, waist circumference and hip circumference were measured in the morning on an empty stomach. Waist circumference was measured at the midpoint of the line connecting the lower rib cage and the skeleton, and hip circumference was measured at the level of the greater trochanter of the femur. BMI and waist to hip ratio (WHR) were calculated. BMI = body weight (kg)/height (m)$^2$, WHR = waist circumference (cm)/hip circumference (cm). Venous blood was collected both after fasting overnight and 2 h later during a standard oral glucose tolerance test. Parameters were measured before administration of exenatide, 3 months and 6 months after administration. Plasma glucose and serum lipids were detected using a Roche Cobas8000 analyzer (Roche, Basel, Switzerland) with standard laboratory methods. Accordingly, the levels of insulin and HbA1c were measured by an electrochemiluminescence assay (Roche, Shanghai, China) and high-performance liquid chromatography (HPLC). The homeostasis model assessment for insulin resistance (HOMA-IR) and homeostasis model assessment for beta cell function (HOMA-B) were calculated using the formula: HOMA-IR = fasting insulin (mU/L)×fasting plasma glucose (mmol/L)/22.5; homeostasis model assessment HOMA-B = 20×fasting serum insulin (FINS)/(FPG-3.5) (23).

## Genotyping

Genomic DNA was extracted from peripheral whole blood leukocytes with a DNA extraction kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. The allelic discrimination of *PPARD* rs2016520 and rs3777744 was performed by SNaPshot assay (Genesky Biotechnologies Inc., Shanghai, China) with the standard protocol (24).

## Definition of the response to exenatide

The guideline of National Institute for Health and Care Excellence (NICE) define T2DM patients who have a ≥1.0% reduction in HbA1c or a ≥3% reduction in body weight after 6 months of treatment with GLP-1 agonists as the treatment response group, and those who do not meet these criteria are defined as Non-responders (25). Based on UK prospective diabetes study (UKPDS)results, a 1% decrease in HbA1c was associated with a 37% reduction in the risk of diabetic microvascular complications and a 21% reduction in diabetes-related end points (26). Since diabetes complications are closely related to HbA1c level, this study mainly evaluated HbA1c changes after exenatide treatment, and defined those T2DM patients whose HbA1c decreased ≥1.0% or endpoint HbA1c < 7.0% after 6 months of exenatide treatment as Responders. Those who do not meet the above criteria are Non-responders.

## Cell culture

The human hepatoma cell line (HepG2) was obtained from the Type Culture Collection of the Chinese Academy of Sciences

(Shanghai, China), cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco BRL, USA) and were placed in a humidified incubator containing an atmosphere of 5% $CO_2$ at 37°C. The insulin-resistant cell model was induced using the previous method (27). HepG2 cells were allowed to attach for 12 h and then serum-starved for 8 h. HepG2 cells were incubated with fresh medium containing 1% FBS, $10^{-3}$ mmol/L insulin (Wanbang Biologic & Medicinal Co., Ltd., Jiangsu, China) for 6 h. Subsequently, the medium was exchanged with medium containing 1% FBS, $10^{-6}$ mmol/L insulin and Exendin-4 ($10^{-1}$ mmol/L, Sigma). Cells were incubated in this medium for 12 h.

## Gene transfection

The transfection of plasmids (6 μg) was carried out using Lipofectamine 3000 Transfection Reagent (Invitrogen, USA) according to the manufacturer's instructions. The ratio of plasmids (μg) and transfection reagents (μL) was 3:4 (27–29). The cells were harvested 48 h after transfection.

## Glucose uptake tests of HepG2 cells

Glucose content was determined by the enzymatic method of the diagnostic kit using 10 μL of medium (Nanjing Jiancheng Bioengineering Inst, China). Data were expressed as extracellular glucose consumption (nmol/mg protein) and calculated as follows: [before extracellular glucose content (nmol) - after extracellular glucose content (nmol)]/mg cellular protein (27), which was measured using the Nanodrop 2000 spectrophotometer (Thermo, USA).

## Western blot analysis for protein levels

Western blot analysis was performed as previously described (28), and the antibodies were applied at concentrations according to the manufacturer's instructions. Actin served as the loading control. Bands were quantified using Image J software. Anti-Actin (AP0060, Bioworld, USA), anti-PPARδ (101562-AP, Proteintech, USA) and anti-GLP-1R (DF7750, Affinity Biosciences, USA) were used in our study.

## Real-time quantitative RT-PCR

Total RNA in HepG2 cells was isolated using Trizol Reagent (15596-026, Invitrogen, USA) according to the manufacturer's instructions, and the steps of RT-PCR were carried out as described previously (29, 30). Data were normalized to internal control β-actin mRNA. Primers were designed and synthesized by Sangon Biotech (Shanghai, China). We used the primer pair 5′-TCTGGAATGGTCTGGAGTGGTCTG-3′ (forward) and 5′-GCCTTGAAGCAGTCCTGTAGAGATC-3′ (reverse) for human PPARδ, 5′- GCAAAGACCTGTACGCCAAC -3′ (forward) and 5′- AGTACTTGCGCTCAGGAGGA -3′ (reverse) for human β-actin, 5′-CCTCCAGATGTCCCCTCCAGATG-3′ (forward) and 5′-CTAAGTGTGCCGCTGCTCCTTC-3′ (reverse) for mouse PPARδ, 5′-AGAGGGAAATCGTGCGTGAC-3′ (forward) and 5′-CAATAGTGATGACCTGGCCGT-3′ (reverse) for mouse β-actin, 5′-GTTCCCCTGCTGTTTGTTGT-3′ (forward) and 5′-CTTGGCAAGTCTGCATTTGA-3′ (reverse) for human GLP-1R.

## Statistical analysis

All data were expressed as mean ± standard deviation (Mean ± SD) or percentage as appropriate. Statistical analyses were performed using SPSS software (version 13.0 for Windows; SPSS Inc., Chicago, IL, USA). Chi-square test was used to compare the Hardy-Weinberg equilibrium, allele frequency and genotype distribution among different groups. Linkage disequilibrium (LD) among SNPs was estimated in subjects using Haploview version 3.2. The two-sample t-test was used to compare the baseline characteristics between T2DM patients and healthy subjects. The paired Student's t-test was applied for evaluating the parameters between the two groups before and after exenatide treatment. For parameters of normal distribution, two-sample t-test was used for comparison between two groups, and one-way analysis of variance (ANOVA) was used for comparison among multiple groups. Parameters with nonnormal distribution were analyzed by the Mann-Whitney U-test or the Kruskal-Wallis test. ANOVA for repeated measurement was used to compare the parameters collected at different treatment time points of the same patient. Statistical power was calculated by power calculator software (http://www.ncss.com). In the experimental study, differences between treated and control results were compared using one-way ANOVA with a Tukey-Kramer post-test for multiple comparisons or unpaired t-test. Two-sided tests were used for all analyses, and $P < 0.05$ indicated statistically significant.

# Results

## Allelic frequency analysis

In the present study, *PPARD* rs2016520 and rs3777744 variants were genotyped in 300 patients with T2DM (196 male and 104 female) and 200 healthy controls (139 male and 61 female) (Table 1). The frequency of the A allele at the *PPARD* rs3777744 locus was lower in patients with T2DM than in healthy controls (57.00% vs. 62.75%, $P = 0.004$), whereas the *PPARD* rs2016520 variant, there was no significant difference in allele frequencies between the two groups.

TABLE 1 Comparison of genotype and frequencies of *PPARD* variants between healthy controls (n = 200) and patients with type 2 diabetes mellitus (T2DM) (n = 300).

| Genotypes | Healthy subjects (frequency, %) | T2DM patients (frequency, %) | *P* value |
|---|---|---|---|
| *PPARD* rs2016520 | | | |
| TT | 106 (53.00) | 148 (49.33) | |
| TC | 83 (41.50) | 125 (41.67) | |
| CC | 11 (5.50) | 27 (9.00) | 0.324 |
| *Alleles* | | | |
| T | 295 (73.75) | 421 (70.17) | |
| C | 105 (26.25) | 179 (29.83) | 0.565 |
| *PPARD* rs3777744 | | | |
| AA | 84 (42.00) | 95 (31.67) | |
| AG | 95 (47.50) | 150 (50.67) | |
| GG | 21 (10.50) | 55 (17.67) | 0.014 |
| *Alleles* | | | |
| A | 263 (62.75) | 340 (57.00) | |
| G | 137 (37.25) | 260 (43.00) | 0.004 |

The genotype distributions of rs2016520 ($P = 0.105$) and rs3777744 ($P = 0.171$) SNPs were in Hardy-Weinberg equilibrium. Assessment of the LD between the variants using our control subjects revealed a relatively low disequilibrium between rs2016520 and rs3777744 (D' = 0.110).

## Assessment of clinical characteristics in T2DM patients with different rs2016520 and rs3777744 genotypes

In the present study, the baseline clinical characteristics of 300 T2DM patients with different rs2016520 and rs3777744 genotypes were analyzed (Tables 2, 3). There were no significant differences in gender, age, postprandial plasma glucose (PPG), HbA1c, postprandial serum insulin (PINS), HOMA-B, triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) between different genotype groups. However, significant differences were observed among patients with different genotypes of *PPARD* rs2016520 in terms of BMI ($P = 0.000$), WHR ($P = 0.001$) and TG ($P = 0.006$) (Supplementary Figure S2). Compared to patients with the AA genotype, patients with the rs3777744 risk G allele had noticeably higher WHR ($P = 0.000$), PPG ($P = 0.011$), FINS ($P = 0.004$) and HOMA-IR ($P = 0.000$) levels (Supplementary Figure S3).

## Effects of the rs2016520 and rs3777744 variants on the efficacy of exenatide in T2DM patients

After 6 months of exenatide treatment, the BMI, WHR, FPG, PPG, HbA1c, HOMA-IR, total cholesterol (TC), TG, and LDL-C values of patients with T2DM were significantly decreased, but the levels of FINS and HOMA-B increased, compared with the pretreatment values (Supplementary Table S1).

Our data also showed that patients with genotype TT at *PPARD* rs2016520 had poor efficacy of exenatide monotherapy with respect to HOMA-IR than C allele carriers (Supplementary Table S2 and Figure 1). Moreover, patients with *PPARD* rs3777744 AG + GG genotypes had attenuated efficacy of exenatide monotherapy with respect to WHR, FPG, HbA1c and HOMA-IR compared with AA genotype carriers (Supplementary Table S2 and Figure 2).

## Association of the rs2016520 and rs3777744 variants with response rate to exenatide treatment

In order to assess the association of the *PPARD* genetic variants with the response rate to exenatide treatment in the present study, the genotypes and allele frequencies were analyzed in the Responder and Non-responder groups (Table 4). According to predetermined exenatide response criteria, *PPARD* rs3777744 A allele carriers exhibited higher response rate to exenatide treatment ($P = 0.007$); AA allele homozygotes had the highest response rate (84.00%), while AG heterozygous and GG homozygous had 61.36% and 54.55%, respectively ($P = 0.022$). No significant effects of *PPARD* rs2016520 variant on exenatide therapy were observed.

To further determine the correlation between *PPARD* variant and improvement in HbA1c after exenatide treatment, a multiple linear regression model was used, with the dependent variable being the decrease in HbA1c after 6 months of exenatide treatment and the independent variables being age, gender, baseline BMI, baseline WHR, baseline HbA1c, rs2016520 and rs3777744. The results showed that the improvement of HbA1c after exenatide treatment was significantly correlated with baseline HbA1c and rs3777744, and the difference between *PPARD* rs3777744 AG+GG genotype and AA genotype in the improvement of HbA1c was statistically significant ($P = 0.009$). Higher the baseline HbA1c was, more significantly the HbA1c improved after 6 months of exenatide treatment ($P = 0.000$) (Table 5).

## Expression of PPARδ in liver tissues of *db/db* mice and in an insulin-resistant HepG2 cell model

To clarify the effect of insulin resistance on the expression level of PPARδ, we used RT-PCR and Western blot to detect the expression level of PPARδ in the liver tissues of *db/db* mice and in an insulin-resistant HepG2 cell model. When compared with the *db/m* group, the mRNA and protein levels of PPARδ in the

TABLE 2 The baseline characteristics in type 2 diabetes mellitus (T2DM) patients with various *PPARD* rs2016520 genotypes before treatment with exenatide (n=300).

| Parameters | *PPARD* rs2016520 genotype | | | Overall *P* value | Adjusted *P* value | | |
|---|---|---|---|---|---|---|---|
| | TT | TC | CC | | TT to TC | TC to CC | TT to CC |
| N (male/female) | 148 (96/52) | 125 (80/45) | 27 (20/7) | 0.600 | | | |
| Age (years) | 49.68 ± 12.88 | 50.08 ± 12.88 | 48.04 ± 14.27 | 0.760 | 1.000 | 1.000 | 1.000 |
| BMI (kg/m$^2$) | 27.16 ± 2.67 | 28.35 ± 4.10 | 29.69 ± 3.80 | 0.000 | 0.014 | 0.195 | 0.001 |
| WHR | 0.93 ± 0.06 | 0.96 ± 0.07 | 0.98 ± 0.08 | 0.001 | 0.014 | 0.488 | 0.007 |
| FPG (mmol/L) | 9.68 ± 2.54 | 10.21 ± 2.55 | 10.62 ± 2.31 | 0.091 | 0.254 | 1.000 | 0.233 |
| PPG (mmol/L) | 14.78 ± 3.86 | 15.70 ± 4.30 | 16.06 ± 3.53 | 0.099 | 0.182 | 1.000 | 0.388 |
| HbA1c (%) | 9.09 ± 1.63 | 9.28 ± 1.76 | 9.42 ± 1.71 | 0.508 | 1.000 | 1.000 | 1.000 |
| FINS (mU/L) | 12.81 ± 8.28 | 14.32 ± 12.37 | 11.53 ± 7.04 | 0.296 | 0.659 | 0.585 | 1.000 |
| PINS (mU/L) | 43.59 ± 32.83 | 42.53 ± 45.55 | 33.48 ± 15.79 | 0.439 | 1.000 | 0.604 | 0.779 |
| HOMA-IR | 5.39 ± 3.97 | 6.27 ± 5.09 | 5.65 ± 4.15 | 0.269 | 0.321 | 1.000 | 1.000 |
| HOMA-B | 52.70 ± 51.27 | 55.49 ± 77.35 | 33.70 ± 18.49 | 0.251 | 1.000 | 0.294 | 0.430 |
| TG (mmol/L) | 2.60 ± 1.86 | 2.17 ± 1.53 | 3.42 ± 3.27 | 0.006 | 0.200 | 0.007 | 0.125 |
| TC (mmol/L) | 5.13 ± 1.27 | 5.03 ± 1.39 | 5.29 ± 1.60 | 0.626 | 1.000 | 1.000 | 1.000 |
| HDL-C (mmol/L) | 1.10 ± 0.30 | 1.12 ± 0.75 | 1.02 ± 0.20 | 0.681 | 1.000 | 1.000 | 1.000 |
| LDL-C (mmol/L) | 3.01 ± 0.88 | 2.96 ± 0.97 | 2.88 ± 0.92 | 0.771 | 1.000 | 1.000 | 1.000 |

BMI, body mass index; WHR, waist to hip ratio; FPG, fasting plasma glucose; PPG, postprandial plasma glucose; $HbA_{1c}$, hemoglobin $A_{1c}$; FINS, fasting serum insulin; PINS, postprandial serum insulin; HOMA-IR, homeostasis model assessment for insulin resistance; HOMA-B, homeostasis model assessment for beta cell function; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; and LDL-C, low-density lipoprotein-cholesterol.

TABLE 3 The baseline characteristics in type 2 diabetes mellitus (T2DM) patients with various *PPARD* rs3777744 genotypes before treatment with exenatide (n=300).

| Parameters | *PPARD* rs3777744 genotype | | | Overall *P* value | Adjusted *P* value | | |
|---|---|---|---|---|---|---|---|
| | AA | AG | GG | | AA to AG | AG to GG | AA to GG |
| N (male/female) | 95 (66/29) | 150 (92/58) | 55 (38/17) | 0.346 | | | |
| Age (years) | 50.46 ± 13.46 | 50.37 ± 12.77 | 46.55 ± 12.40 | 0.136 | 1.000 | 0.184 | 0.224 |
| BMI (kg/m$^2$) | 27.52 ± 3.10 | 27.94 ± 3.47 | 28.36 ± 4.26 | 0.355 | 1.000 | 1.000 | 0.474 |
| WHR | 0.88 ± 0.06 | 0.94 ± 0.07 | 0.95 ± 0.07 | 0.000 | 0.000 | 1.000 | 0.000 |
| FPG (mmol/L) | 9.76 ± 2.26 | 10.16 ± 2.71 | 9.92 ± 2.53 | 0.483 | 0.237 | 0.547 | 0.722 |
| PPG (mmol/L) | 13.82 ± 2.87 | 15.22 ± 3.26 | 15.38 ± 4.08 | 0.011 | 0.018 | 1.000 | 0.052 |
| HbA1c (%) | 9.08 ± 1.62 | 9.17 ± 1.58 | 9.51 ± 2.06 | 0.310 | 1.000 | 0.592 | 0.417 |
| FINS (mU/L) | 11.02 ± 5.81 | 13.58 ± 10.45 | 16.59 ± 13.64 | 0.004 | 0.152 | 0.170 | 0.003 |
| PINS (mU/L) | 36.60 ± 27.02 | 45.36 ± 44.24 | 43.46 ± 33.29 | 0.230 | 1.000 | 1.000 | 0.848 |
| HOMA-IR | 4.84 ± 2.52 | 5.94 ± 3.62 | 6.84 ± 4.98 | 0.000 | 0.021 | 0.119 | 0.001 |
| HOMA-B | 43.60 ± 51.32 | 51.32 ± 52.57 | 69.16 ± 98.61 | 0.050 | 1.000 | 0.201 | 0.045 |
| TG (mmol/L) | 2.75 ± 2.33 | 2.36 ± 1.64 | 2.43 ± 1.92 | 0.306 | 0.394 | 1.000 | 0.994 |
| TC (mmol/L) | 5.09 ± 1.35 | 5.16 ± 1.47 | 4.99 ± 0.99 | 0.720 | 1.000 | 1.000 | 1.000 |
| HDL-C (mmol/L) | 1.06 ± 0.24 | 1.09 ± 0.32 | 1.18 ± 1.09 | 0.340 | 1.000 | 1.000 | 0.440 |
| LDL-C (mmol/L) | 2.96 ± 0.89 | 2.98 ± 0.96 | 2.99 ± 0.87 | 0.969 | 1.000 | 1.000 | 1.000 |

BMI, body mass index; WHR, waist to hip ratio; FPG, fasting plasma glucose; PPG, postprandial plasma glucose; $HbA_{1c}$, hemoglobin $A_{1c}$; FINS, fasting serum insulin; PINS, postprandial serum insulin; HOMA-IR, homeostasis model assessment for insulin resistance; HOMA-B, homeostasis model assessment for beta cell function; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; and LDL-C, low-density lipoprotein-cholesterol.

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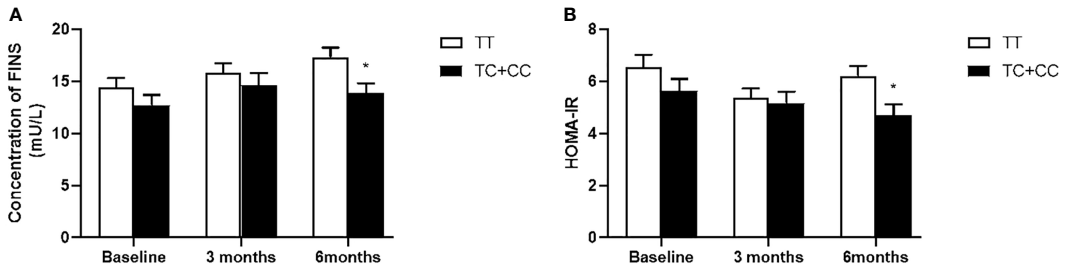


FIGURE 1
Comparison of fasting serum insulin (FINS) **(A)** and homeostasis model assessment for insulin resistance (HOMA-IR) **(B)** between T2DM patients with the TT genotype (n = 62) and those with TC and CC genotypes (n = 43) genotypes of *PPARD* rs2016520 in T2DM patients before, at 3 months and at 6 months of exenatide treatment. Data are expressed as the mean ± SE, **P*<0.05 compared with TT genotype group (n= 105).

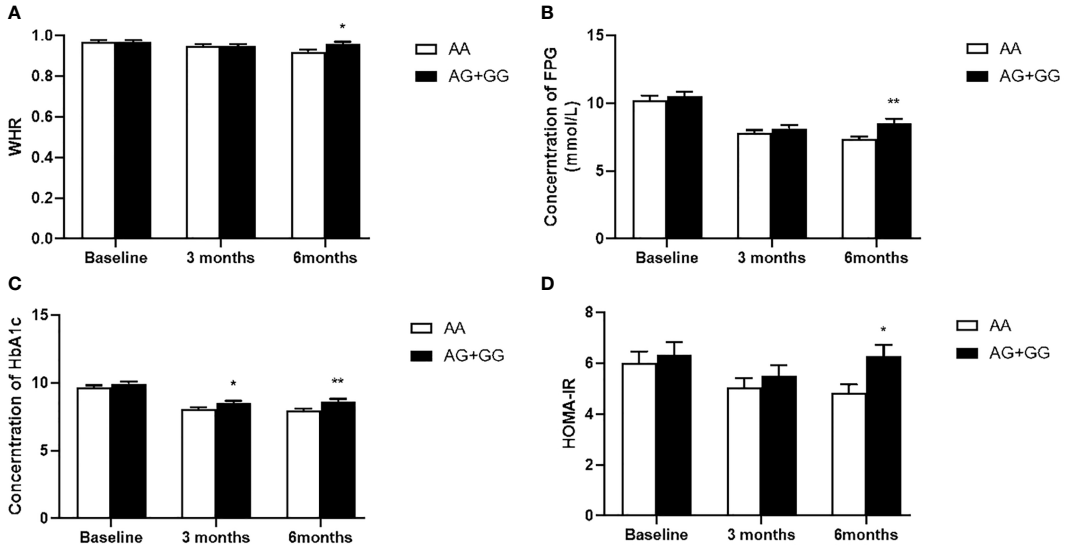


FIGURE 2
Comparison of waist to hip ratio (WHR) **(A)**, fasting plasma glucose (FPG) **(B)**, hemoglobin A1c (HbA1c) **(C)** and homeostasis model assessment for insulin resistance (HOMA-IR) **(D)** between T2DM patients with the AA genotype (n = 50) and those with AG and GG genotypes (n = 55) genotypes of *PPARD* rs3777744 in T2DM patients before, at 3 months and at 6 months of exenatide treatment. Data are expressed as the mean ± SE, **P*<0.05 and ***P*<0.01 compared with AA genotype group (n = 105).

TABLE 4 Genotype and allele distributions between responders and non-responders of *PPARD* rs2016520 and rs3777744 variants (n = 105).

| | Genotype | | | *P* value | Allele frequency | | *P* value |
|---|---|---|---|---|---|---|---|
| *PPARD* rs2016520 | TT | TC | CC | | T | C | |
| Responder (%) | 42 (67.74%) | 25 (75.75%) | 8 (80.00%) | | 109 (69.43%) | 41 (77.36%) | |
| Non-responder (%) | 20 (32.26%) | 8 (24.24%) | 2 (20.00%) | 0.584 | 48 (30.57%) | 12 (22.64%) | 0.182 |
| *PPARD* rs3777744 | AA | AG | GG | | A | G | |
| Responder (%) | 42 (84.00%) | 27 (61.36%) | 6 (54.55%) | | 111 (77.08%) | 39 (59.09%) | |
| Non-responder (%) | 8 (16.00%) | 17 (38.64%) | 5 (45.45%) | 0.022 | 33 (22.92%) | 27 (40.91%) | 0.007 |

TABLE 5 Multiple linear regression analysis of HbA1c improvement after 6 months of exenatide treatment (n = 105).

| Variables | β | 95% CI | *P* value |
|---|---|---|---|
| Age (years) | 0.011 | (-0.003, 0.024) | 0.630 |
| Sex (male/female) | 0.096 | (-0.006, -0.023) | 0.261 |
| Baseline BMI (kg/m$^2$) | 0.045 | (-0.025, 0.114) | 0.205 |
| Baseline WHR | 0.709 | (-3.477, 4.865) | 0.736 |
| Baseline HbA1c (%) | -0.553 | (-0.705, -0.402) | 0.000 |
| rs2016520 | 0.196 | (-0.155, 0.546) | 0.271 |
| rs3777744 | 0.432 | (0.233, 1.321) | 0.009 |

BM, body mass index; WHR, waist to hip ratio; $HbA_{1c}$, hemoglobin $A_{1c}$.

liver tissues of *db/db* group were significantly lower ($P < 0.05$) (Supplementary Figures S4A, B). The results of *in vitro* experiments showed that in HepG2 cells, there was no difference between PPARδ expression in the solvent control group (SC) and the negative control group (NC), but both mRNA and protein expression of PPARδ were significantly lower in the insulin resistant group (IR) ($P < 0.05$) (Supplementary Figures S4C, D). According to the *in vivo* and *in vitro* results, PPARδ expression levels were significantly decreased in insulin resistant HepG2 cells and in liver tissue of *db/db* mice.

## PPARδ controls exenatide therapeutic efficacy in insulin resistance by regulating the expression of GLP-1R

To further validate the mechanism by which PPARδ regulates exenatide therapeutic efficacy, relationship between PPARδ activity and GLP-1R expression was investigated. The silencing PPARδ plasmid and silencing negative control (siNC) plasmid were transfected in HepG2 cells, and the knockdown efficiency of the three plasmids (siPPARδ-1, siPPARδ-2 and siPPARδ-3) was detected. The RT-PCR and western blot results showed the most significant reduction in the siPPARδ-3 group (Supplementary Figure S5). Therefore, the silencing plasmid named siPPARδ-3 was finally selected for the follow-up experiment.

As shown in Figure 3A, both PPARδ agonists (GW501516) and Exendin-4 significantly increased the ratio of glucose uptake in IR HepG2 cells, and the efficacy of Exendin-4 was significantly enhanced after treatment with GW501516. In addition, the expression of GLP-1R in IR HepG2 cells were up-regulated markedly after administration of PPARδ agonist (Figure 3B, C). As predicted, the knockdown of PPARδ hindered glucose metabolism and down-regulated the expression of GLP-1R in HepG2 cells (Figure 4). These results indicated that PPARδ plays a pivotal role in insulin resistance through regulating the expression of GLP1-R and influences the ability of exenatide to agonize GLP-1R to improve insulin resistance.

# Discussion

In the current study, we evaluated the potential impact of two SNPs (rs2016520 and rs3777744) of *PPARD* on the outcomes of exenatide in treating Chinese patients with T2DM. Genetic variants associated with T2DM susceptibility or metabolism-related indicators have been proved in several pharmacogenomic studies to be important factors in the efficacy of exenatide (18–20). The results of this study revealed that patients with the TT genotype of *PPARD* rs2016520 or the G allele of rs3777744 may have a weaker response to exenatide therapy, indicating that the *PPARD* genotype can be used as a predictor of response to exenatide. Therefore, our findings support the prior generation of genotyping and screening of patients with T2DM for gene-directed individualized dosing in the clinical application of exenatide. Moreover, we also observed the critical role of PPARδ in regulation of the expression of GLP-1R, the receptor for exenatide, and effects on insulin resistance.

Common SNPs in *PPARD* are associated with an increased risk of impaired glucose tolerance, fasting glucose elevation and insulin resistance in populations with diverse ethnic backgrounds including Chinese, Korean and Mexican (5–8). In the present study, we focused on genetic variants in PPARD and found that the risk G allele of rs3777744 (43.00%) in patients with T2DM had a higher frequency than that in the control group ($P < 0.01$) and it was higher than the data from the 1000 Genomes (36.60%) (31). In contrast, *PPARD* rs2016520 did not show a significant association with T2DM in our subjects, but the frequency of the C allele of rs2016520 (29.83%) was lower than that in the data from the 1000 Genomes (36.60%) (31). Comparison of our findings with the 1000 Genomes data showed that *PPARD* rs2016520 and rs3777744 showed dramatically different allele frequencies in different ethnic populations. The major reasons for this discrepancy may be the differences in specific ethnic groups and exposure to environmental factors. The main drivers of this variation may be due to differentiation in specific ethnic groups and the influence of exposure to diverse environments. The current study also displayed higher BMI and WHR values in T2DM patients with *PPARD* rs2016520 TC and CC genotypes, which indicated a potential contribution of the genetic variant to the prevalence of overweight and obesity in T2DM patients (Table 2 and Supplementary Figure S2). In contrast, WHR, PPG, FINS and HOMA-IR were higher in patients with the rs3777744 risk G allele compared with those carrying the A allele (Table 3 and Supplementary Figure S3). Potent associations have been determined between genetic variation in the *PPARD* gene and elevated susceptibility to T2DM, as well as obesity and insulin resistance. In addition, PPARδ is involved in regulating energy metabolism in liver, skeletal muscle and adipose tissue, and it is a mechanism by which *PPARD* gene variants lead to obesity and insulin resistance (32, 33).

The known involvement of PPARδ in insulin resistance, either directly or indirectly suggests that *PPARD* gene variants

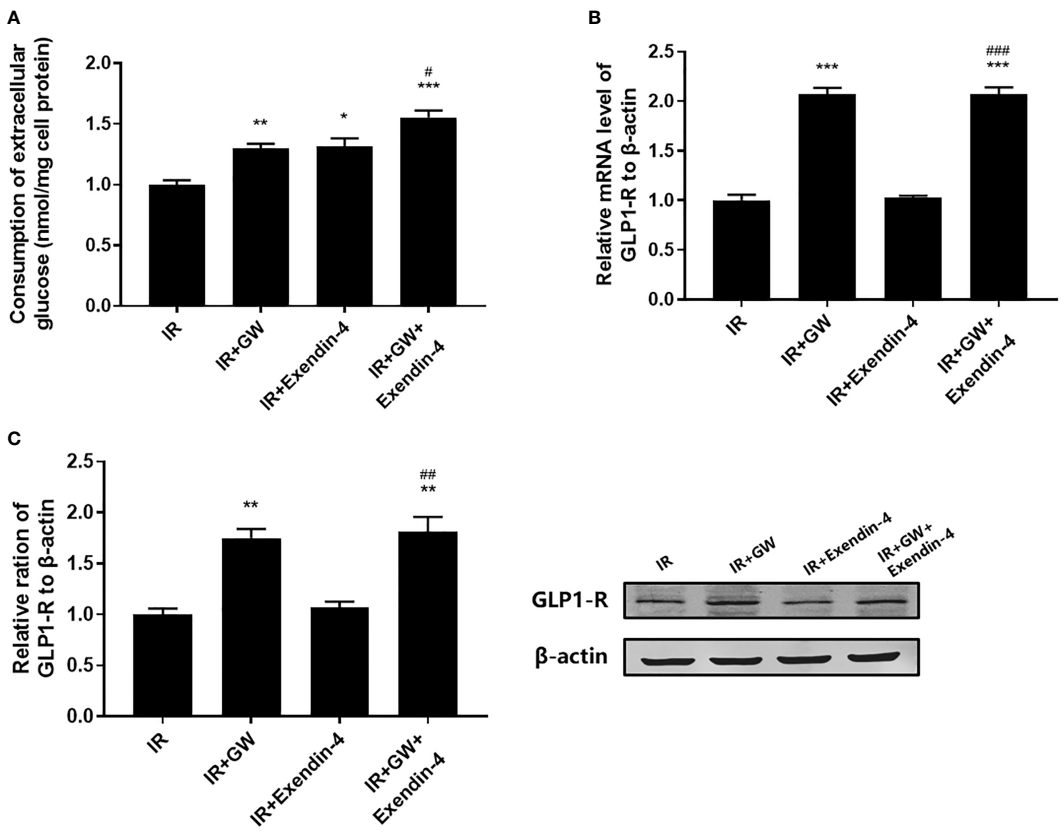


FIGURE 3
Effect of PPARδ agonists (GW501516) on the exenatide therapeutic efficacy by regulating the expression of GLP-1R. **(A)** Enzymatic methods were used to assay for glucose. **(B)** The mRNA level of GLP-1R was measured by RT-PCR. **(C)** The relative protein expression level of GLP-1R was measured by Western blot. Data are expressed as the mean ± SE, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with IR. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with IR+Exendin-4.

may account for individual differences in the clinical efficacy of exenatide. There is no evidence from previous studies that patients with specific *PPARD* gene variants have better or worse clinical efficacy of exenatide. In this study, we observed that the FINS and HOMA-IR levels of the subjects with at least one C allele of the *PPARD* rs2016520 were significantly lower than those with the TT genotype after 6 months of treatment, which suggested that the *PPARD* rs2016520 TT genotype conferred the poor exenatide response through a reduction of insulin resistance, as measured by HOMA-IR. PPARδ is engaged in glucose and lipid metabolism in the liver and exerts insulin-sensitizing effects, thereby improving hepatic insulin resistance (34). Therefore, our findings suggest that *PPARD* rs2016520 may affect the biological function of PPARδ, thereby influenceing the therapeutic effects of exenatide on improving insulin resistance of patients with T2DM.

The carriers of G alleles at rs3777744 exhibited higher levels of WHR, FPG, HbA1c and HOMA-IR compared to individuals with the AA genotype following 6 months of exenatide treatment, potentially accounting for the lower failure rate of exenatide therapy among the AA homozygotes. Additional analysis of metabolic features revealed that the G allele of rs3777744 had a detrimental effect on FPG and HOMA-IR, suggesting that an increased FPG and HbA1c may be caused, at minimum partially, by the negative effects of insulin resistance. Regarding rs3777744, which is located at intron2 of *PPARD* at chromosome 6p21.31, it is confirmed that the rs3777744 G allele is associated with cardiovascular disease risk in the Chinese population, though the underlying mechanism is unclear (35). A wealth of studies support the notion that cardiovascular disease is related to both insulin resistance and the compensatory hyperinsulinemia associated with insulin resistance (36).

Therefore, the genetic variants of *PPARD* might be responsible for interindividual differences in exenatide response. The underlying mechanisms leading to these findings remain unclear, but the effects of the *PPARD* rs3777744 G allele on IR, PPG and HbA1c need to be explored further to clarify the underlying mechanisms.

We hypothesize that the underlying mechanism by which *PPARD* variants contribute to individual differences in the

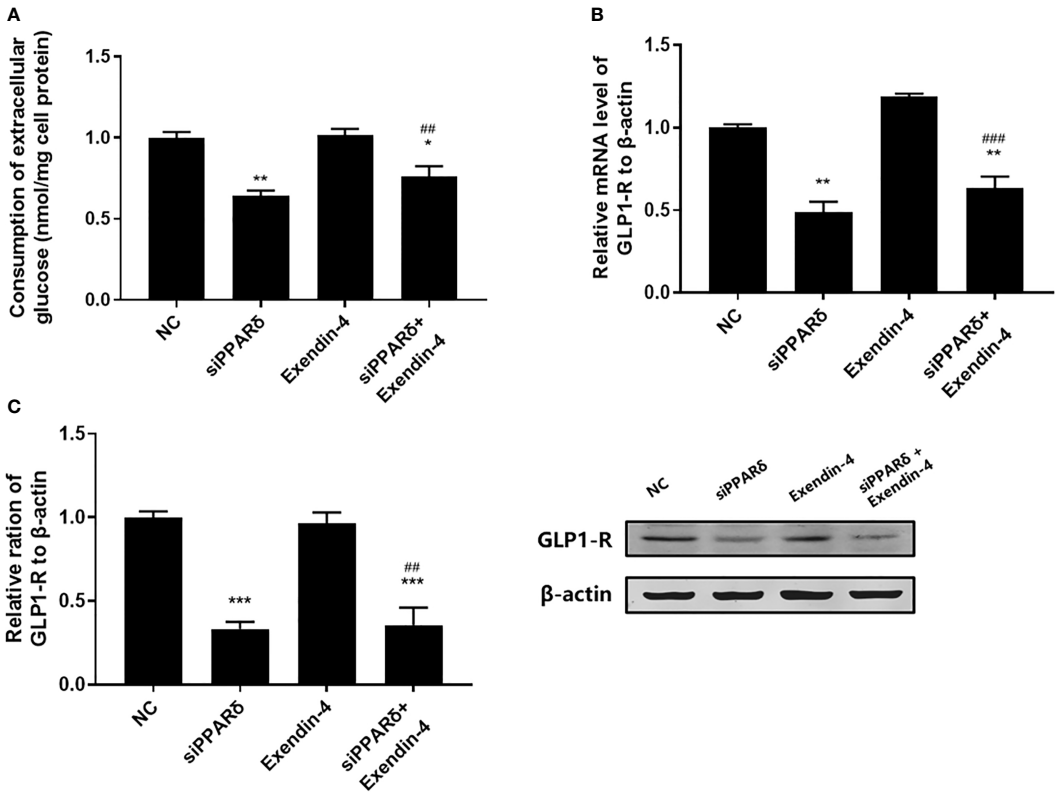


FIGURE 4
Effect of PPARδ knockdown on the exenatide therapeutic efficacy by regulating the expression of GLP-1R. **(A)** Enzymatic methods were used to assay for glucose. **(B)** The mRNA level of GLP-1R was measured by RT-PCR. **(C)** The relative protein expression level of GLP-1R was measured by Western blot. Data are expressed as the mean ± SE, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with NC. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with Exendin-4.

development of T2DM and the therapeutic effects of exenatide is that PPARδ regulates the expression and function of GLP-1R. To fully illustrate the role of *PPARD* on exenatide efficacy and its associated pathways in IR, we established the insulin resistance model in HepG2 cells. Interestingly, evidence showed that SNPs in intron regions can affect gene function mainly by influencing splice site activity, while SNPs in the 5'-UTR may affect its binding to transcription factors, thus affecting protein expression and function (37). Therefore, we hypothesized that the regulation of *PPARD* gene expression in cells could resemble the genetic variation and functional changes of *PPARD* in the clinic. PPARδ is essentially a class of ligand-dependent transcriptional regulators, and GLP-1R is the pharmacological target of exenatide. We identified potential PPARδ binding sites by analyzing the GLP-1R promoter through the JASPAR database (Supplementary Figure S1). Therefore, we hypothesized that PPARδ regulates the level and function of GLP-1R and may be the underlying mechanism of *PPARD* gene variants. In this study, we found that the activation of PPARδ enhanced the uptake of extracellular glucose and the protein expression of GLP-1R in IR HepG2 cells, suggesting that PPARδ played a critical role in the regulation of insulin signaling pathways under pathological conditions. To further assess whether PPARδ is involved in the efficacy of exenatide, we examined the efficacy of exenatide on glucose uptake in the cellular level. In an IR cell model, we observed that activation of PPARδ potentiated the therapeutic benefits of exenatide in IR and the expression level of GLP-1R were significantly elevated. Consistent with the results of clinical trial, these data strongly suggest that exenatide increases insulin sensitivity in the liver, which could be further strengthened by overexpression of PPARδ. On the other hand, we knocked down PPARδ on HepG2 cells and performed the mentioned experiments and observed the opposite effect, corroborating with the above results.

Meanwhile, several shortcomings of this study need to be considered when interpreting our findings. First, the sample size is relatively small, which results in restricted statistical power and low frequency of mutant phenotypes, this may have led us to miss some meaningful results. The current study had an estimated 81-97% power (for $\alpha$=0.05) to detect the difference in the parameters. Consequently, further detailed studies with

expanded sample size are warranted to validate the effects of *PPARD* variants on exenatide efficacy. Second, individual differences are the product of interactions between multiple genetic and environmental factors. In the present study, different exenatide responses were found to be associated with *PPARD* gene variants, but it cannot be excluded that other susceptibility genetic variants are also involved in individual differences of exenatide therapeutic efficacy. Therefore, we will estimate the co-effect of multiple loci on the efficacy of exenatide in the following studies. Third, the different locations of *PPARD* rs2016520 (in the 5'-UTR) and rs3777744 (located in intron2) have made it more difficult to carry out functional studies. We only explored the regulatory effect of PPARδ on GLP-1R and its effect on exenatide efficacy, however, the mechanism of SNPs in *PPARD* influence on PPARδ has not been elucidated.

In conclusion, the *PPARD* variants appear to be associated with the therapeutic response to exenatide in patients with T2DM. There may be a link between PPARδ and GLP-1R in the diabetic condition, which may be the molecular mechanism by which *PPARD* gene variants influence T2DM risk, insulin resistance and clinical response to exenatide. Therefore, the *PPARD* risk mutations may serve as exenatide response predictors based on PPARδ regulating GLP-1R expression and mediating insulin resistance. More detailed pharmacogenetic and functional studies are needed to elucidate the exact effects of *PPARD* variants on exenatide therapeutic efficacy, which is necessary to lay the foundation for a more precise and patient-tailored therapy for T2DM.

## Data availability statement

The data presented in the study are deposited in the https://submit.ncbi.nlm.nih.gov/subs/biosample/SUB11888229/overview (NCBI) repository, accession number SUB11888229.

## Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Affiliated Hospital of Xuzhou Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Experiment Ethics Review Committee of Xuzhou Medical University.

## Author contributions

JS contributed to conception of the article, data acquisition, statistical analysis, result interpretation, manuscript drafting and approved the final version. TW and XY designed the experiments and revised the paper. NL, RH, YY, KX, QL and TY helped with the experiments. HL contributed to data acquisition, and revised the paper. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.949990/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Predicted GLP-1R binding motif site sequence from the database JASPAR.

SUPPLEMENTARY FIGURE 2
Baseline levels of body mass index (BMI) **(A)**, waist to hip ratio (WHR) **(B)** and triglyceride (TG) **(C)** in T2DM patients with TT (n = 148), TC (n = 125) and CC (n = 27) genotypes of *PPARD* rs22016520. *$P < 0.05$ and **$P<0.01$ compared with the TT genotype group, $^{\#\#}P<0.01$ compared with the TC genotype group (n = 300).

SUPPLEMENTARY FIGURE 3
Baseline levels of waist to hip ratio (WHR) **(A)**, postprandial plasma glucose (PPG) **(B)**, fasting serum insulin (FINS) **(C)** and homeostasis model assessment for insulin resistance (HOMA-IR) **(D)** in T2DM patients with AA (n = 95), AG (n = 150) or GG (n = 55) genotypes of *PPARD* rs3777744. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001compared with the AA genotype group (n = 300).

SUPPLEMENTARY FIGURE 4
The expression of PPARδ decreased in IR HepG2 cell model and *db/db* mice. **(A)** The mRNA level of PPARδ in HepG2 cells was measured by RT-PCR. Data are expressed as the mean ± SE, n = 3. **(B)** The relative protein expression level of PPARδ in HepG2 cells was measured by Western blot. Data are expressed as the mean ± SE, n = 3. $^{***}P < 0.001$ compared with NC. **(C)** The mRNA level of PPARδ in *db/db* mice was measured by RT-PCR. Data are expressed as the mean ± SE, n = 6. **(D)** The relative protein expression level of PPARδ in *db/db* mice was measured by Western blot. Data are expressed as the mean ± SE, n = 6. $^{***}P < 0.001$ compared with *db/m*.

SUPPLEMENTARY FIGURE 5
Validation of PPARδ plasmid knockdown efficiency in HepG2 cells. **(A)** The mRNA level of PPARδ in HepG2 cells was measured by RT-PCR. **(B)** The relative protein expression level of PPARδ in HepG2 cells was measured by Western blot. Data are expressed as the mean ± SE, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with siNC.

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# Tandem mass tag-based proteomic profiling revealed potential therapeutic targets and mechanisms of liraglutide for the treatment of impaired glucose tolerance

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**Objective:** Based on the tandem mass tag (TMT) technique, our study investigated the potential therapeutic targets of Liraglutide (LIRA) on streptozotocin (STZ) induced impaired glucose tolerance (IGT) in rats and discuss the biological mechanism of the drug against IGT.

**Methods:** 10 rats were randomly selected from 31 male wistar rats of specific pathogen free (SPF) grade as control group and fed with conventional chow, offered the remaining rats a high fat and high sugar (HFSD) diet combined with an intraperitoneal injection of STZ to establish the IGT model, and excluded 2 non-model rats. Specifically, the model rats were randomly divided into Model group (n=10) and LIRA group (n=9). In addition, the LIRA group was subcutaneously injected with 0.06 mg/kg LIRA, during which the metabolic parameters including body weight and fasting blood glucose were recorded. After 8 weeks, samples were taken under anesthesia. Then, the cell morphology was observed using HE staining, and immunofluorescence was performed on the pancreatic tissues of the three groups of rats. Besides, the expression of differential proteins in pancreatic tissues of the three groups of rats was determined by the TMT proteomic labeling. Subsequently, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) biological function analysis were performed on the intersection of Model and LIRA differential proteins.

**Results:** LIRA could not only significantly reduce blood glucose levels but also improve islet cell morphology and function in IGT rats. Among the differential proteins between the model group and the blank group, 44 were reversed after LIRA treatment, of which 14 were up-regulated, while 30 were down-regulated, including PPIF, MPRIP, CYP51, TXNL1, BCL-2, etc. (FC>1.1

or<0.909, $P$<0.05). According to the GO and KEGG analysis results, it was related to biological processes such as fatty acid metabolism and adipocyte generation, which involved multiple signaling pathways regulating the function of islet cells, such as MAPK, PI, Ras, FcγR, and unsaturated fatty acids, and pyruvate metabolism.

**Conclusion:** To sum up, LIRA participated in anti-IGT therapy through regulation of multiple target proteins and biological functions. This study is of great reference for further exploring the mechanism of action of LIRA at the protein level of IGT.

KEYWORDS

**liraglutide (LIRA), impaired glucose tolerance (IGT), tandem mass tag (TMT) proteomics, protein targets, mechanism of action**

## 1. Introduction

Impaired glucose tolerance (IGT) refers to a special metabolic state, in which the fasting blood glucose (FBG) is below 7.0 mmol/L, and the 2h blood glucose after oral glucose tolerance test (OGTT) is between 7.8-11.0 mmol/L. Moreover, it is a necessary stage in the development of Type 2 Diabetes Mellitus (T2DM) as well as the important stage where T2DM can be controlled and reversed (1). At present, IGT becomes more common in young people. According to the IDF statistics, people suffering from impaired glucose tolerance are estimated to amount to 374 million in 2019 and may reach 454 million by 2030 and 548 million by 2045 (2). It is noteworthy that the etiology and pathogenesis of IGT have not been fully elucidated, and islet β-cell dysfunction and insulin resistance (IR) may be the main causes (3). Generally speaking, drugs including metformin and acarbose are mainly used to treat IGT. However, patients who take these two medicines tend to experience adverse reactions such as liver and kidney toxicity, stomach pain, and diarrhea (4). Therefore, it is of great significance to seek supplementary and alternative medicines to prevent and treat IGT.

It has been found that glucagon-like peptide-1 (GLP-1) can act on islet β-cells in a glucose concentration-dependent manner, stimulate insulin secretion, inhibit glucagon secretion, and reduce blood sugar (5). After endogenous GLP-1 is degraded by dipeptidyl peptidase-4 (DPP-IV), its biological activity is rapidly lost, and its half-life in blood is merely 1-2 min, so that it cannot be used to treat IGT (6). In fact, 97% of the amino acid sequence of LIRA, a novel long-acting GLP-1 receptor agonist, is overlapped with endogenous GLP-1. LIRA replaces one amino acid in the molecular structure of endogenous GLP-1 and adds a 16-carbon fatty acid chain, which prevents LIRA from being degraded and extends its half-life to 13h (7). As revealed by some studies, LIRA could lower blood sugar levels, improve islet cell function, and decrease the risk of adverse cardiovascular events (8). Besides, myocardial ischemic contracture was reduced in myocardial ischemic-reperfused injury rats in the IGT + LIRA group (9). In comparison to the Model, IGT mice after LIRA intervention had higher C-peptide levels after the OGTT test, indicating that LIRA can improve islet β-cell function in IGT rats (10). In addition, serum Fetuin-B expression was significantly decreased in IGT patients after LIRA intervention (11). At present, the precise target of LIRA in the treatment of IGT remains unclear. Our novelty lies in that we pay attention to the IGT through TMT technology, with a view to focus on the early development of T2DM. Proteomics can identify and analyze changes in proteins at the overall cellular level. By detecting abnormal changes in protein expression, it can not only clarify the occurrence and development of diseases and potential targets of drugs, but also provide comprehensive information on cell dynamics (12). As a high-throughput screening technology, TMT technology uses multiple isotopic reagents to label the N-terminal or lysine side chain group of protein polypeptides and conducts high-precision tandem mass spectrometry, which has been commonly applied in quantitative proteomics in recent years (13).

Through analyzing TMT marker proteomics, this study constructed an IGT rat model to explore the potential protein targets of LIRA in the treatment of IGT as well as its possible biological pathways, providing new research ideas and directions for the prevention and treatment of IGT and T2DM.

# 2 Methods and materials

## 2.1 Laboratory animals

In this research, 31 five-week-old male wistar rats of SPF grade weighing (130 ± 10)g were purchased from Jinan Pengyue Biological Breeding Co., Ltd. (#1107261911005606), and then raised in the Experimental Animal Center of Shandong University of Traditional Chinese Medicine (Temperature: 18-22°C, humidity: 60%-70%, light-dark cycle: 12h, 5 animals/cage, SPF level feeding, free food and water). During the experiment, all the procedures were strictly implemented in accordance with Chinese guidelines, including *Laboratory Animal - Requirements of Environment and Housing Facilities* (GB14925-2001) and *Guidelines for Humane Handling of Laboratory Animals* (MOST 2006a). Apart from that, all the animal experiments were approved by the Animal Ethics Committee of Shandong University of Traditional Chinese Medicine (No. SDUTCM20190520001).

## 2.2 Model preparation and grouping

After 31 male Wistar rats of SPF grade were adaptively fed for 1 week, 10 rats were randomly selected as Control group, and the rest were fed with HFSD (19093212, Keao Xieli Feed Co., Ltd., Beijing), which were composed of 67% conventional feed, 10.0% lard, 20.0% sucrose, 2.5% sodium cholate, and 2.5% cholesterol. Referring to the previous studies and related literature, rats in the model group were given a one-time intraperitoneal injection of STZ 15 mg/kg (18883-66-4, Sigma, United States). STZ was dissolved in 0.1 mmol/L sodium citrate (2018010203, Zhiyuan Company, Tianjin) and citric acid (2018-09-16, Dingshengxin Company, Tianjin) buffer, pH=4.4, final concentration was 4%, used right after it was ready, and used up within 20min), whereas the rats of Control group were injected intraperitoneally with citrate buffer (pH=4.4) at a dose of 0.1ml/L. In the wake of 72 hours, blood was collected from the tail vein of the rats, and the FPG and 2hPG values of the rats were measured by OGTT test. If FPG of three OGTT tests performed on different days were all <7.0mmol/L and 7.8mmol/L ≤ 2hPG <11.1mmol/L, it could be considered that the modeling was successful. What's more, two non-model rats were excluded, and the remaining model rats were randomly divided into Model group (n=10) and LIRA group (n=9). More specifically, LIRA rats were subcutaneously injected with 0.06 mg/kg LIRA (J20180026, Novo Nordisk, Denmark) (14), whereas Control and Model rats were given equal volumes of normal saline. In addition, the body weight and OGTT test results (portable blood glucose meter: Johnson & Johnson, USA) of each group were measured at a fixed time every week. For the rats in each group, they were allowed to drink water freely and fasted for 12 hours after administration for 8 weeks (Figure 1).

## 2.3 HE staining to observe the morphology and structure of islet cells in three groups of rats

An appropriate amount of rat pancreatic tissue was fixed in 10% formaldehyde solution for 12 h, dehydrated in ethanol, embedded in paraffin (after immersed in xylene), cut into slices with a thickness of 4 μm, dried at 45°C, and then HE stained and

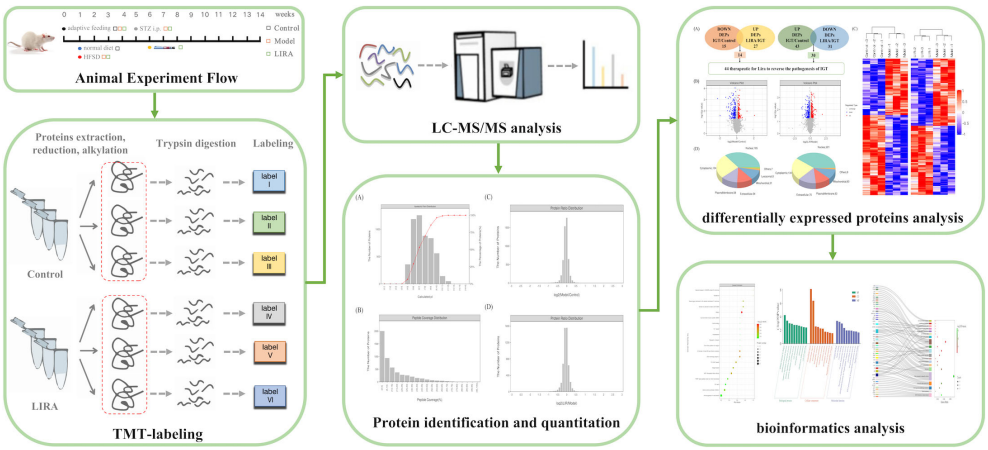


FIGURE 1
Flow chart of animal experiments. The diagram of TMT marker quantitative proteomics analysis operation mainly included steps such as protein extraction, peptide enzymatic hydrolysis, TMT labeling, chromatographic fractionation, liquid chromatography-tandem mass spectrometry (LC-MS/MS) data collection, data retrieval, GO and KEGG biological enrichment functional analysis, etc. LIRA, Liraglutide; STZ, StrepStozocin; ip, intraperitoneal injection; HFSD, high-fat and high-sugar diet.

mounted. Afterwards, the light microscope was used to observe the morphology and distribution of islet cells.

## 2.4 Immunofluorescence detection of the localization and distribution of islet α/β cells

Paraffin sections (LEICA company, Germany) were deparaffinized and rehydrated in graded alcohols. In addition, antigens were retrieved, autofluorescence quencher was added, and bovine serum albumin was added for incubation. Furthermore, primary antibodies anti-insulin mouse mAb (GB13121, Servicebio, China) and anti-glucagon rabbit pAb (GB13097, Servicebio, China) were added dropwise to the sections, incubated overnight, washed, incubated with corresponding secondary antibodies, and washed. Besides, DAPI (Solarbio, C0060) staining solution was added dropwise, and anti-fade mounting medium was adopted to mount the slides. Then, images were observed and collected under the fluorescence microscope (Leica DM2500 microscope: Leica, Germany).

## 2.5 Quantitative proteomic analysis of TMT markers

This study utilized TMT quantitative proteomics technology to analyze the expression of differential proteins in the three groups (Figure 1), randomly selected three pancreatic samples from each group, extracted the protein by SDT lysis buffer (4% (w/v) SDS, 100mM Tris/HCl pH7.6, 0.1M DTT), and quantified the BCA. What's more, filter-aided proteome preparation (FASP) was used for trypsin digestion, followed by peptide quantification (OD280). Afterwards, the peptides were labeled according to the instructions of the TMT labeling kit (Thermo Company) and fractionated using the High pH Reversed-Phase Peptide Fractionation Kit. Beyond that, each sample was separated using the HPLC liquid phase system Easy nLC (Thermo Scientific, USA), and then mass spectrometry analysis was made using a Q-Exactive mass spectrometer (Thermo Scientific, USA). Furthermore, data were identified and quantified using software Mascot 2.2 and Proteome Discoverer 1.4. With fold change (FC)>1.1 folds (up-regulation>1.1-fold or down-regulation<0.909-fold) and <0.05 as the standard, the number of differentially expressed up-regulated and down-regulated proteins between groups was screened out for cluster analysis. Based on the R package, two dimensions of sample and protein expression were classified, and a hierarchical clustering heat map was generated. Apart from that, CELLO was used to perform Subcellular localization prediction. Notably, the intersection of the up-regulated differentially expressed protein targets (Model vs Control) and the down-regulated differentially expressed protein targets (LIRA vs Model) is the specific target that is up-regulated after the onset of IGT and improved after LIRA treatment. At the same time, the intersection of the down-regulated differentially expressed protein targets (Model vs Control) and the up-regulated targets (LIRA vs Model) is the specific target that is down-regulated after the onset of IGT and improved after the LIRA treatment. In order to further explore the functions of the proteins in cells, the subcellular localization analysis of all differentially expressed proteins was performed using the subcellular structure prediction software CELLO. Beyond that, GO and KEGG functional annotations were performed on the differential proteins between LIRA and Model to clarify the biological processes and signaling pathways involved in LIRA treatment of IGT. Moreover, Blast2 GO was used to perform GO annotation analysis on the target protein set, and KAAS software was adopted to conduct KEGG pathway annotation on the target protein set.

## 2.6 Statistical analysis

All the data were statistically analyzed by SPSS 24.0 software and the experimental data were expressed by $\bar{X} \pm S$ (mean ± sd). In addition, the SNK test was used for the comparison between groups, the paired t-test was performed for the comparison within groups, and the chi-square test ($\chi^2$) was applied to analyze count data. $P<0.05$ was considered statistically significant.

# 3 Results

## 3.1 Comparison of body weight, FBG, and 2hPG among the three groups of rats (n=9)

After 8 weeks of intervention, the body weight of the Model rats increased in comparison to that of the Control rats ($P<0.05$), whereas the body weight of LIRA rats was lower than that of Model rats ($P<0.05$), indicating that the body weight of IGT rats could be reduced by LIRA. Compared with Control rats, the FPG of Model rats increased ($P<0.05$). In comparison to the Model rats, FPG of LIRA rats decreased, but the difference was not statistically significant ($P>0.05$). After treatment, in comparison to the Control rats, 2hPG of Model rats increased ($P<0.05$), and 2hPG decreased after LIRA intervention ($P<0.05$), indicating that LIRA could reduce 2hPG in IGT rats (Figure 2A).

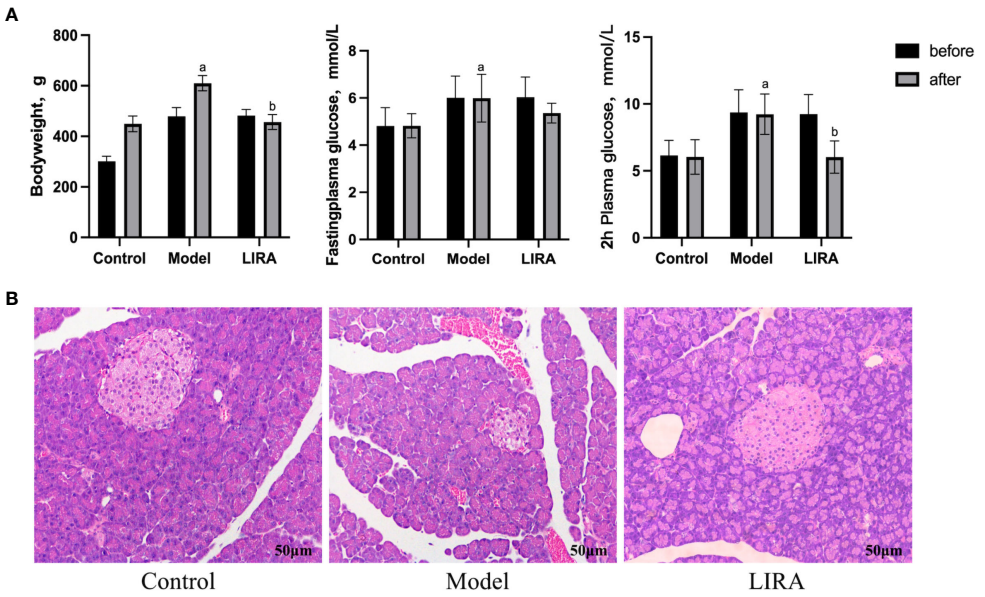


FIGURE 2
**(A)** Changed of body weight/FPG/2hPG of rats in each group before and after intervention (n=9). Compared with Control group, [a] $P<0.05$; Compared with Model group, [b] $P<0.05$. **(B)** The morphology and structure of islets were observed by HE staining (200×). From left to right are control, model and LIRA (n=3).

## 3.2 HE staining to observe the morphology and structure of rat pancreas (n=3)

The islet tissue of the Control rats had clear borders, with the surrounding tissue being evenly distributed. Besides, the islet cells were arranged regularly, with uniform size and a large number. In comparison to the pancreatic tissue of the Control rats, the pancreatic tissue of the IGT rats which were established by HFSD combined with STZ had unclear borders. Meanwhile, the islet cells were disordered, irregular in size and shape, and few in number. In comparison to IGT rats, the islets of LIRA rats had clear borders and regular round shapes. Besides, the islet cells were arranged relatively neatly, and their number increased relatively (Figure 2B).

## 3.3 Immunofluorescence observation of islet cells (n=3)

The islet β cells of the Control rats were aggregated in the center of islet, with a large number and uniform distribution, islet α cells were distributed around β cells, with a few numbers and uniform distribution. In comparison to the Control group, the number of islet β cells in the Model rats decreased, and the distribution of islet cells were uneven, the number of islet α cells increased, the distribution of the islet α cells was uneven. Compared to Model group, the number of islet β cells in LIRA rats increased and the islet β cells aggregated in the center of islet, the islet α cells, which were small in number, were mainly distributed in the periphery of the islet (Figure 3).

## 3.4 Analysis of TMT proteomics results (n=3)

### 3.4.1 Quality control analysis

In this experiment, the quality deviations of all identified peptides were mainly distributed within 10 ppm, indicating that the quality deviations were small, and the identification results were accurate and reliable (Figure 4A). As shown by the MASCOT analysis, MS2 had an ideal MASCOT score, in which more than 51.31% of the peptides scored more than 20 points, and the median peptide scored 21 points, revealing that the quality of MS experimental data was credible (Figure 4B). What's more, the abundance ratio of most proteins approached 1 in the two groups of equally labeled samples (Figures 4C, D). According to the above results, this experiment has maintained a good quality deviation throughout the whole process, and the experimental data collected are credible.

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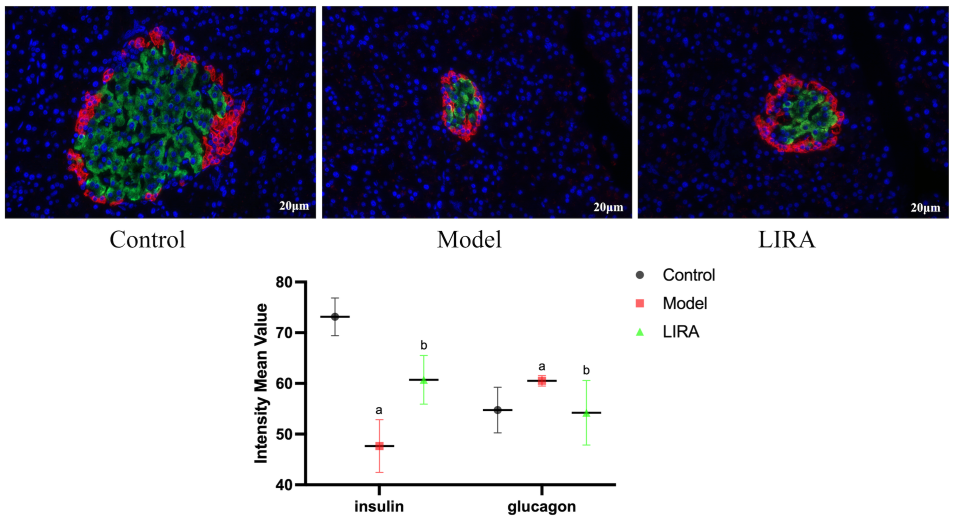


FIGURE 3
Immunofluorescence observation of islet α/β cells (400X light microscope). The red fluorescence were insulin staining, representing α cells; the green fluoresces were glucagon staining, representing β cells (n=3). Compared with Control group, [a] $P<0.05$; Compared with Model group, [b] $P<0.05$.

### 3.4.2 Identification of differential protein DEPs between groups and potential protein targets of LIRA

According to the screening criteria (FC>1.1 or <0.909, $P<0.05$), the proteomic analysis of the three groups of samples revealed that 43 differential proteins of Model/Control were up-regulated, and 30 were down-regulated after drug intervention (Figure 5A). To be specific, the target proteins are PAPSS1, DAD1, CIAO1, DLGAP4, DCAF11, NSA2, ADAM10, PDCD10, PTPA, G-septin, Rmdn2, LRRC59, MRPS2, HMOX2, BET1L,

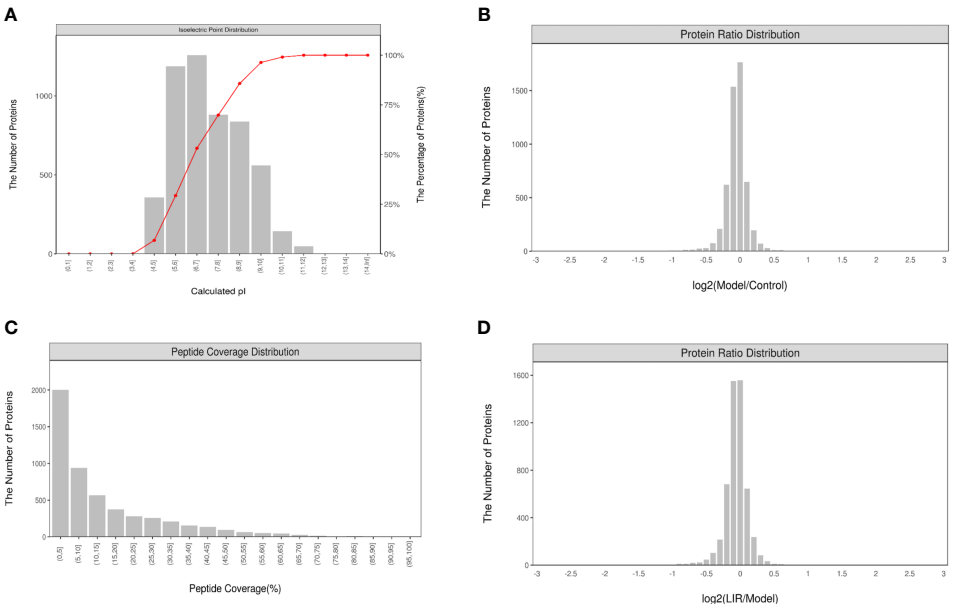


FIGURE 4
Quality control analysis and characterization of peptides (n=3). **(A)** Peptides identified distribution; **(B)** Protein sequence coverage distribution; **(C)** Distribution of protein abundance ratios between Model and Control. **(D)** Distribution of protein abundance ratios between LIRA and Model. The data showed that the quality control analysis and the experimental data were reliable.

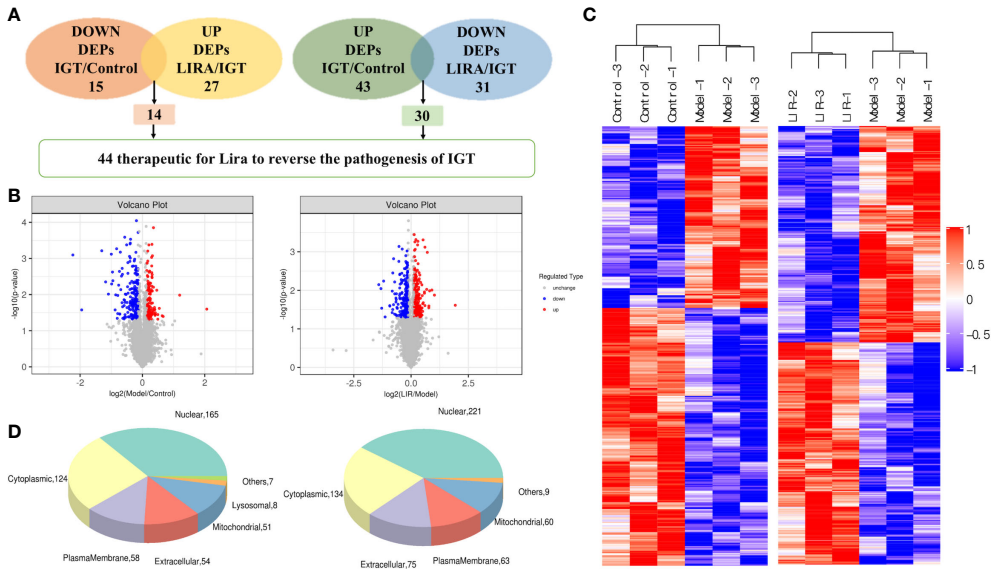


FIGURE 5
**(A)** The number of differentially expressed proteins among three groups (n=3). **(B)** Volcano plots of significantly differentially expressed protein targets among the three groups. dots in the volcano plot represent differential proteins, red represents up-regulation, green represents down-regulation, and black represents differential proteins that did not meet the screening conditions and were excluded. The left figure represents Model/Control differential protein screening, and the right figure represents LIRA/Model differential protein screening. **(C)** Heat map of significantly differentially expressed proteins between groups. the horizontal axis represents the sample number, the vertical axis represents the differential protein, the color represents the relative expression of the differential protein, and clustering analysis was performed on the differential proteins with similar expression. **(D)** The subcellular localization of differentially expressed proteins among the three groups. The left panel showed the subcellular localization of Model/Control differential proteins; the right panel showed the subcellular localization of LIRA/Model differential proteins.

ECI1, YIF1B, AGFG2, ATP5MF, SUB1, UGT2B10, ACOT2, BCL2, MEN1, PSMb6L, ATG2B, Serpina3c, Afm, LYRM4, and ENY2 respectively. In the wake of LIRA treatment, 15 differential proteins of Model/Control were down-regulated (Table 1) and 14 were reversed, namely TBC1D13, TCEAL9 (WBP5), PPIF, ME2, MKK6 (MAP-KK6), MPRIP (MRIP), SELENOF, CYP51, HDAC6, SLC6A9, DNASE2, SelT, FKBP8, TXNL1 (Table 2). Based on the comprehensive comparison, the potential protein targets of LIRA in IGT model rats were the above 44 differential proteins, suggesting that LIRA regulates the differential expression of these 44 proteins, so as to treat IGT model rats, regulate islet cell function, and lower blood sugar levels. Apart from that, volcano plots are drawn to clearly display significant differences in proteins between groups (Figure 5B), and clustering analysis was performed on target proteins in the form of Heat-map (Figure 5C).

## 3.5 Biological function analysis (n=3)

It can be observed from the data that most of the differential proteins between the two groups were located in the nucleus and cytoplasm (221, 134/165, 124), followed by extracellular matrix, plasma membrane and mitochondria (75, 63, 60/54, 58, 51), and some differential proteins were localized in other places (e.g., endoplasmic reticulum and Golgi). In addition, 3 of the differentially expressed proteins between LIRA/Model were located in lysosomes (Figure 5D).

According to the protein domain analysis, the domain of DEPs was mainly enriched in the Vesicle transport -SNARE protein N-terminus, Transferrin, C2 domain in Dock180 and Zizimin proteins, and Globin, etc (Figure 6). Then, GO analysis indicated that the action pathways of LIRA in the treatment of IGT mainly include apoptosis regulation process (GO:0043066), gene expression regulation (GO:0010628), positive regulation of GTPase activity (GO:0043547), cell chemotaxis (GO:0060326), regulation of Ras active factor (GO:0005088), etc. (Figure 7). As suggested by the results of KEGG analysis, the anti-IGT signaling pathway of LIRA may not only be related to the activation of MAPK, FcεRI, GnRH, P13K and other signaling pathways, but also be associated with FcγRI-mediated phagocytosis, apoptosis, ubiquitinated proteolysis, unsaturated fatty acid metabolism and aldosterone metabolism (Figure 8).

10.3389/fendo.2022.1031019

TABLE 1 Down-regulated pancreatic DEPs in LIRA compared with Model rats (n=3).

| Accession | Protein Name | Model/Control | | LIRA/Model | |
|---|---|---|---|---|---|
| | | FC | P. value | FC | P. value |
| ENSRNOP00000071489 | PAPSS1 | 1.183 | 0.002 | 0.908 | 0.007 |
| ENSRNOP00000012233 | DAD1 | 1.203 | 0.017 | 0.907 | 0.030 |
| ENSRNOP00000017603 | CIAO1 | 1.184 | 0.004 | 0.906 | 0.011 |
| ENSRNOP00000034166 | DLGAP4 | 1.148 | 0.015 | 0.901 | 0.001 |
| ENSRNOP00000074435 | DCAF11 | 1.179 | 0.000 | 0.898 | 0.002 |
| ENSRNOP00000022138 | NSA2 | 1.184 | 0.029 | 0.897 | 0.034 |
| ENSRNOP00000073306 | ADAM10 | 1.299 | 0.001 | 0.895 | 0.022 |
| ENSRNOP00000013585 | PDCD10 | 1.111 | 0.043 | 0.890 | 0.027 |
| ENSRNOP00000024914 | PTPA | 1.215 | 0.005 | 0.888 | 0.045 |
| ENSRNOP00000010491 | G-septin | 1.203 | 0.014 | 0.887 | 0.045 |
| ENSRNOP00000008045 | Rmdn2 | 1.155 | 0.015 | 0.886 | 0.013 |
| ENSRNOP00000004941 | LRRC59 | 1.286 | 0.000 | 0.885 | 0.017 |
| ENSRNOP00000013548 | MRPS2 | 1.106 | 0.026 | 0.884 | 0.010 |
| ENSRNOP00000075102 | HMOX2 | 1.131 | 0.021 | 0.884 | 0.023 |
| ENSRNOP00000017684 | BET1L | 1.119 | 0.019 | 0.871 | 0.024 |
| ENSRNOP00000011784 | ECI1 | 1.126 | 0.001 | 0.868 | 0.008 |
| ENSRNOP00000053771 | YIF1B | 1.153 | 0.030 | 0.861 | 0.028 |
| ENSRNOP00000074192 | AGFG2 | 1.174 | 0.026 | 0.857 | 0.037 |
| ENSRNOP00000029426 | ATP5MF | 1.115 | 0.005 | 0.856 | 0.034 |
| ENSRNOP00000067467 | SUB1 | 1.188 | 0.037 | 0.855 | 0.040 |
| ENSRNOP00000069054 | UGT2B10 | 1.204 | 0.014 | 0.843 | 0.018 |
| ENSRNOP00000013515 | ACOT2 | 1.298 | 0.005 | 0.832 | 0.012 |
| ENSRNOP00000003768 | BCL2 | 1.195 | 0.023 | 0.826 | 0.030 |
| ENSRNOP00000028592 | MEN1 | 1.182 | 0.002 | 0.812 | 0.033 |
| ENSRNOP00000015747 | PSMb6L | 1.398 | 0.031 | 0.765 | 0.037 |
| ENSRNOP00000006131 | ATG2B | 1.348 | 0.035 | 0.728 | 0.015 |
| ENSRNOP00000013896 | Serpina3c | 1.248 | 0.029 | 0.726 | 0.034 |
| ENSRNOP00000057275 | Afm | 1.184 | 0.030 | 0.720 | 0.009 |
| ENSRNOP00000065815 | LYRM4 | 1.364 | 0.010 | 0.717 | 0.020 |
| ENSRNOP00000006402 | ENY2 | 1.449 | 0.042 | 0.643 | 0.025 |

IGT, impaired glucose tolerance; LIRA, Liraglutide; FC, fold change.

# 4 Discussion

IGT is a metabolic disease between normal glucose tolerance and type 2 diabetes mellitus, which may be accompanied by obesity, insulin resistance and other diseases. It is mainly manifested as impaired fasting blood glucose and postprandial blood glucose. In the case of normal glucose regulation, early-phase insulin secretion may become impaired, and further aggravation is very likely to progress to IGT. As IGT progresses to the course of T2DM, islet β-cell function will decrease progressively. Therefore, the key for treating T2DM is to conduct the early intervention in the IGT phase, so as to protect islet β-cells and alleviate β-cell hypofunction. LIRA is a long-acting GLP-1 receptor agonist with 97% homology to native GLP-1. As shown by evidence, LIRA has anti-islet β-cell apoptosis properties and can prolong islet β-cell lifespan in patients with type 2 diabetes mellitus (10). It has been found in the previous study that the miRNA expression profile of STZ-induced diabetic rat pancreatic tissue is abnormal. Beyond that, high-throughput sequencing could identify 9 target miRNAs for LIRA to treat diabetes, involving autophagy, Fox O, PI, HIF-1, and other signaling pathways (15). In this study, TMT proteomics technology was used to screen out 44 potential protein targets of LIRA in the treatment of IGT, including TBC1D13, PPIF, MPRIP, ME2, CYP51, DAD1, PTPA, TXNL1, ATG2B, BCL-2, etc., suggesting that these target proteins may be associated with insulin resistance, islet beta cell insufficiency, etc. At the same time, LIRA can change the expression of the above differential proteins, making them more likely to be in the blank group. That is to say, LIRA improves

TABLE 2 Up-regulated pancreatic DEPs in LIRA compared with Model rats (n=3).

| Accession | Protein Name | Model/Control | | LIRA/Model | |
|---|---|---|---|---|---|
| | | FC | P. value | FC | P. value |
| ENSRNOP00000021431 | TBC1D13 | 0.697 | 0.020 | 2.053 | 0.012 |
| ENSRNOP00000050036 | TCEAL9 | 0.462 | 0.006 | 1.772 | 0.007 |
| ENSRNOP00000014382 | PPIF | 0.800 | 0.027 | 1.349 | 0.003 |
| ENSRNOP00000073241 | ME2 | 0.542 | 0.007 | 1.334 | 0.024 |
| ENSRNOP00000006217 | MKK6 | 0.710 | 0.035 | 1.306 | 0.039 |
| ENSRNOP00000069198 | MPRIP | 0.792 | 0.026 | 1.284 | 0.013 |
| ENSRNOP00000075579 | SELENOF | 0.715 | 0.006 | 1.281 | 0.010 |
| ENSRNOP00000009985 | CYP51 | 0.882 | 0.009 | 1.249 | 0.005 |
| ENSRNOP00000063689 | HDAC6 | 0.890 | 0.023 | 1.236 | 0.003 |
| ENSRNOP00000070161 | SLC6A9 | 0.863 | 0.045 | 1.231 | 0.000 |
| ENSRNOP00000014887 | DNASE2 | 0.899 | 0.050 | 1.192 | 0.028 |
| ENSRNOP00000072567 | SelT | 0.883 | 0.034 | 1.157 | 0.020 |
| ENSRNOP00000074539 | FKBP8 | 0.836 | 0.002 | 1.146 | 0.000 |
| ENSRNOP00000074049 | TXNL1 | 0.873 | 0.012 | 1.113 | 0.027 |

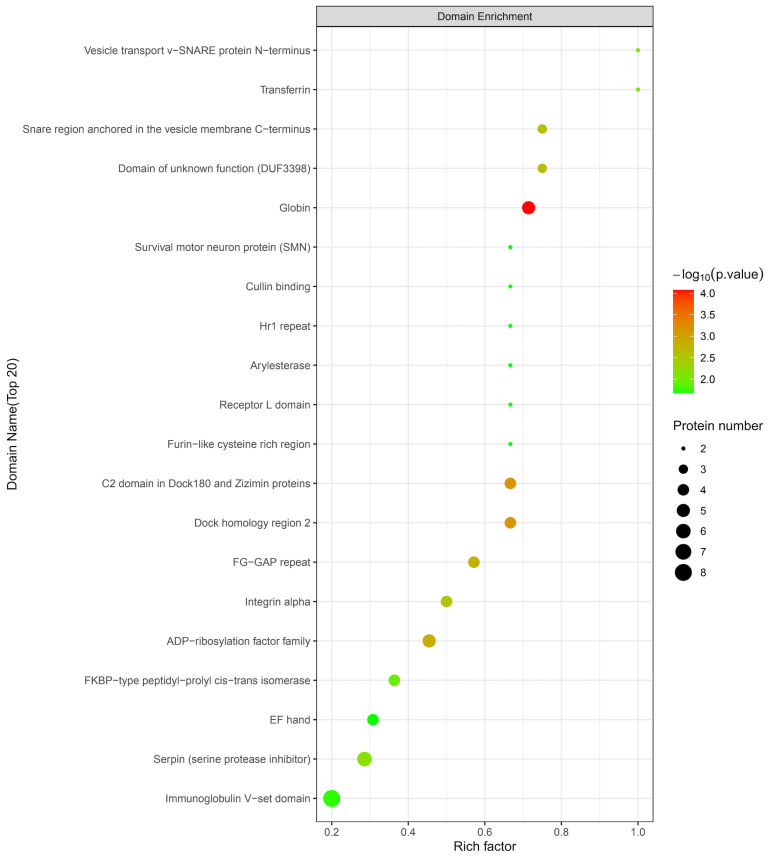


FIGURE 6
LIRA/Model differential protein domain analysis diagram (n=3). The horizontal axis is the rich factor (Rich factor ≤ 1), and the vertical axis represents the statistical results of DEPs under each domain classification. The color of the bubbles indicates the significance of enrichment under the corresponding domain classification, the P value is calculated by Fisher's Exact Test, and the color gradient indicates -log10 (P value). The redder the color, the smaller the P-value, and the higher the significance level of the enrichment under the corresponding domain classification.

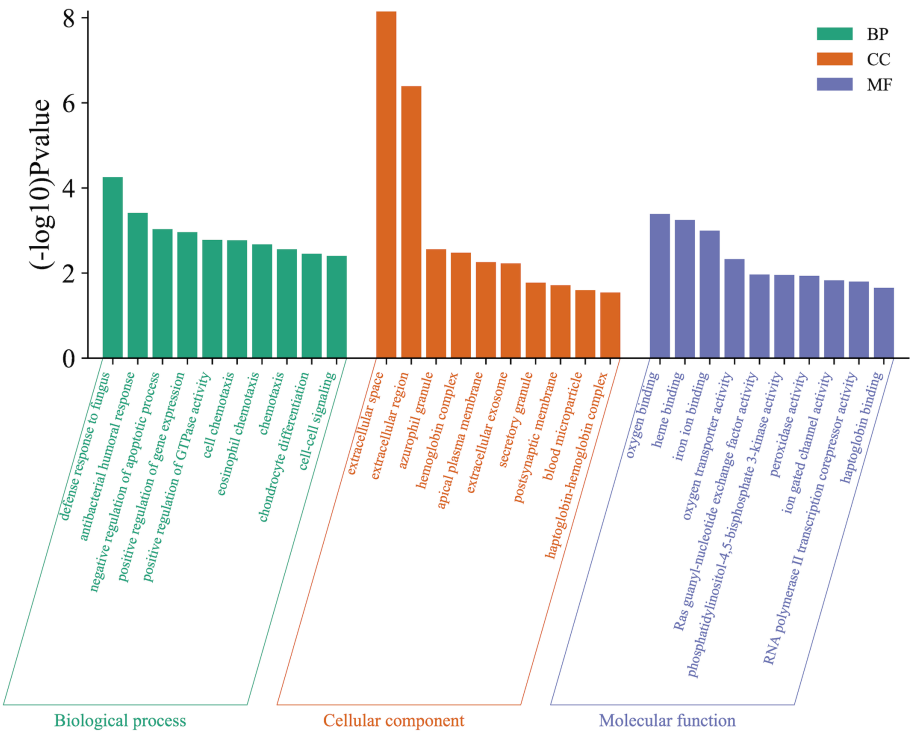


FIGURE 7
GO functional enrichment analysis (n=3). The figure shows the top 10 related enrichment pathways of each GO functional analysis.

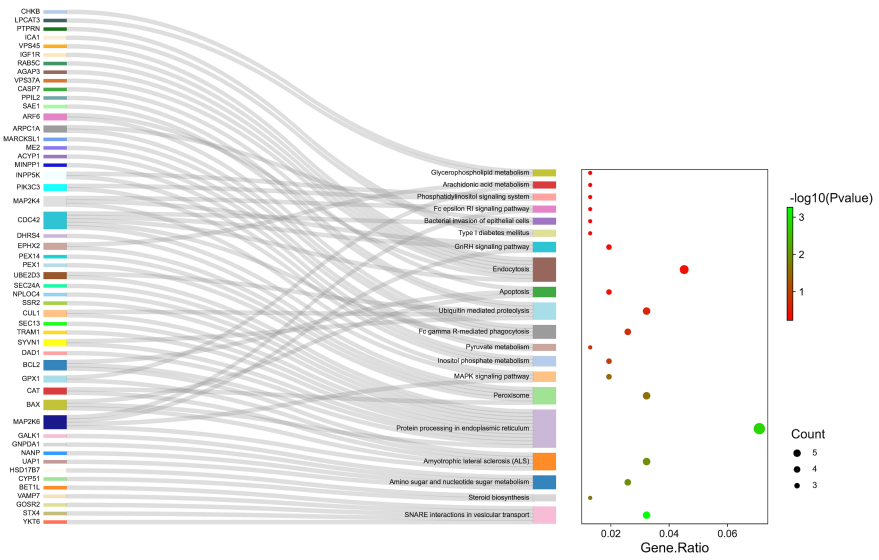


FIGURE 8
KEGG pathway enrichment analysis (n=3). The genes associated with the enrichment pathway are represented on the left. The bubble area size represented the number of genes belonging to the pathway in the target gene, and the bubble color represented the enrichment significance, i.e., the size of the P value. The top 10 pathways enriched by KEGG were shown in figure.

insulin resistance and islet function in IGT rats to a certain extent by affecting glucose transport, apoptosis, lipid metabolism, and autophagy, among other pathways.

According to the previous studies, the expression of the above differential proteins is related to glucose and lipid metabolism, insulin resistance, and insufficiency of islet cell function. For example, it has been confirmed that PPIF is related to FBG levels, insulin sensitivity and islet cell function in the diabetic rats. In the skeletal muscle of diabetic rats, Atp5, Ant1 and PPIF mRNA levels are decreased, while Ant2 mRNA levels are increased (16). In this experiment, it was found that the expression of PPIF in the model group was decreased, and the expression of PPIF was significantly increased after LIRA intervention (P=0.003), revealing that LIRA may reduce blood sugar levels by increasing insulin sensitivity and improving islet cell function in IGT rats. In a clinical study of more than 20,000 people, it had been proved that human vitamin E-binding glycoprotein Afamin was positively associated with HOMA-IR in type 2 diabetes mellitus (β=0.110 [95%CI 0.089-0.132], $P=1.37\times10^{-23}$) (17). In addition, some studies demonstrated that Menin, the protein encoded by MEN1, can interact with various epigenetic mediators to regulate gene transcription, and inhibit pancreatic β-cell proliferation. Meanwhile, Menin improves islet β-cell function and reduces blood sugar levels in diabetic rats (18). Besides, CYP51 has been confirmed to get involved in glucose and lipid metabolism, and astragali powder can significantly up-regulate the expression of this gene in obese rats induced by a high-fat diet, which is consistent with the results of this study (19). Moreover, there is a potential relationship between the nuclear protein Eny2 and insulin secretion (20). Inhibiting the expression of Eny2 induces an increase in the level of incretin. LIRA reduces the level of blood glucose in IGT rats by down-regulating the expression of Eny2 in our study. Therefore, we presumed that the mechanism of action of LIRA may be related to the regulation of the secretion of incretin. On the basis of previous studies, it can be observed the expression of ME2 was elevated in db/db rats, which was reversed after ATR treatment. This finding is consistent with the results of this study (21).

In addition to the above findings, the mechanism by which LIRA treats IGT and improves blood glucose levels in rats may also be related to the regulation of islet cell apoptosis, oxidative stress, and reduction of inflammatory responses. According to the experimental data, LIRA can up-regulate the expression of anti-apoptotic gene DAD1 (22), which was consistent with the results of our study. As pointed out by He C et al., in HFSD-induced diabetic rats, in comparison to the control group, the blood glucose level of rats was significantly decreased after the intervention of 10% Yunvjian medicated serum, and the mechanism may be down-regulation of autophagy protein ATG2B to protect INS-1 cells from glucolipid toxicity-induced apoptosis (FC=0.914). This finding is in line with the trend in this study (23). The data showed that in comparison to the control group, the expression of insulin and apoptosis protein Bcl-2 in the model group decreased, while the insulin level and the expression of Bcl-2 protein increased significantly after probiotic intervention ($P<0.01$). That is to say, LIRA might improve the survival rate of islet cells, inhibit cell apoptosis, and improve islet cell function by regulating the expression of Bcl-2 protein, so that the purpose of anti-IGT can be achieved (24). In fact, Selenoprotein T (SelT) is a redox protein. This study found that LIRA can reverse the expression of SelT in the model group, which may be related to oxidative stress, etc (25). TXNIP, which is involved in the activation of the NLRP3 inflammasome, is closely related to the onset of type 2 diabetes mellitus (26). As claimed by Bai S et al, MMP-2 is related to the progression of chronic diabetes complications, and exosomal circ_DLGAP4 regulates the expression of miR-143 and targets the ERBB3/NF-κB/MMP-2 axis, which triggers the occurrence of diabetic nephropathy (27). In addition, some studies have argued that MRIP is related to vasodilatory function. By combining with MYPI1, it activates the vasodilatory signaling pathway. At the same time, the expression of MRIP decreases in a high-glucose environment, the interaction with MYPI1 diminishes, and the vasodilation function declines, leading to hypertension in diabetic patients (28). In this study, the expression of MRIP in the model group was decreased, and the expression was up-regulated after LIRA intervention, indicating that LIRA can improve the vasodilation function of diabetic rats, and the mechanism may be related to the vasodilatory signaling pathway mediated by insulin resistance. As suggested by previous studies, the ADAM10 and ADAM17 downregulate STZ-induced type 1 diabetes mellitus in rats, and the mechanism is related to atherosclerosis (29).

According to the functional enrichment analysis, the LIRA reduces blood glucose levels in IGT rats and improves islet cell function, which involves multiple signaling pathways such as MAPK, PI, FcγR, FcεRI, as well as unsaturated fatty acids and pyruvate metabolism, apoptosis, and endocytosis. The study found that the natural isoquinoline alkaloid palmatine (PAL) can help improve high-fat diet (HFD)-induced insulin resistance in IGT rats, increase islet β-cell proliferation, and strengthen islet cell function. The mechanism may activate MAPK signaling pathway through the regulation of JNK signal transduction, thereby improving insulin deficiency (30). FcγR and its ligands are closely related to the pathogenesis of obesity and T2DM. As shown by some studies, the FcγR not only blocks insulin-induced phosphorylation of AKT and FOXO1, but also up-

regulates the expression of G6Pase and PEPCK mRNA in a high-glucose environment, which reveals the potential role of FcγR in regulating glycolipid metabolism (31). Beyond that, a Mendelian randomized trial found a positive correlation between type 2 diabetes mellitus and the prevalence of amyotrophic lateral sclerosis (ALS) in Asian populations. As shown by the Bioinformatic analysis of this study, the mechanism of action of LIRA in the treatment of IGT is associated with ALS-related pathways. LIRA may play a potential role in decreasing the prevalence of ALS in patients, and the possible mechanism needs to be further studied (32). In addition, KEGG analysis revealed that the anti-sugar mechanism of LIRA may also be related to the intestinal flora. As confirmed by some studies, LIRA regulates insulin secretion in obese rats by targeting the gut microbiota and gut immune system (33). According to the bacterial 16S rRNA sequencing analysis, among the patients who were treated with LIRA, the alpha diversity of gut microbes was reduced, the distribution of microbiota structure was altered, and the interactions of microbes were changed, suggesting that LIRA may reduce the blood glucose level and improve the inflammatory response of patients with DM through regulation of the intestinal flora, mainly Lactobacillus and Clostridium (34). In short, the above studies show that LIRA improves the function of islet β cells in IGT rats, regulates glucose and lipid metabolism, reduces islet cell apoptosis, and modulates the pharmacological effects of intestinal flora through multiple dimensions and pathways.

## 5 Conclusions

In this experiment, TMT quantitative proteomics technology was first used to analyze the biological process of LIRA in treatment of IGT rats. On this basis, 44 potential protein targets, biological processes, and related signaling pathways were identified. In the subsequent experiments, we will continue studying the above-mentioned target proteins and further explore their related mechanisms of action to provide new research ideas and therapeutic directions for the prevention and treatment of IGT.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

## Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of Shandong University of Traditional Chinese Medicine (No. SDUTCM20190520001).

## Author contributions

JL designed the experiments. QG and CH performed the experiments and wrote the manuscript. SZ and YX performed the bioinformatic analyses. QC and XH provided comments. HL supervised the study. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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# Sodium glucose co-transporter 2 (SGLT2) inhibition via dapagliflozin improves diabetic kidney disease (DKD) over time associatied with increasing effect on the gut microbiota in db/db mice

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**Background:** The intestinal microbiota disorder gradually aggravates during the progression of diabetes. Dapagliflozin (DAPA) can improve diabetes and diabetic kidney disease(DKD). However, whether the gut microbiota plays a role in the protection of DAPA for DKD remains unclear.

**Methods:** To investigate the effects of DAPA on DKD and gut microbiota composition during disease progression, in our study, we performed 16S rRNA gene sequencing on fecal samples from db/m mice (control group), db/db mice (DKD model group), and those treated with DAPA (treat group) at three timepoints of 14weeks\18weeks\22weeks.

**Results:** We found that DAPA remarkably prevented weight loss and lowered fasting blood glucose in db/db mice during disease progression, eventually delaying the progression of DKD. Intriguingly, the study strongly suggested that there is gradually aggravated dysbacteriosis and increased bile acid during the development of DKD. More importantly, comparisons of relative abundance at the phylum level and partial least squares-discriminant analysis (PLS-DA) plots roughly reflected that the effect of DAPA on modulating the flora of db/db mice increased with time. Specifically, the relative abundance of the dominant Firmicutes and Bacteroidetes was not meaningfully changed among groups at 14 weeks as previous studies described. Interestingly, they were gradually altered in the treat group compared to the model group with a more protracted intervention of 18 weeks and 22 weeks. Furthermore, the decrease of *Lactobacillus* and the increase of *norank_f:Muribaculaceae* could account for the differences at the phylum level observed between the treat group and the model group at 18 weeks and 22 weeks.

**Conclusion:** We firstly found that the protective effect of DAPA on DKD may be related to the dynamic improvement of the gut microbiota over time, possibly associated with the impact of DAPA on the bile acid pool and its antioxidation effect.

KEYWORDS

dapagliflozin, diabetes kidney disease (DKD), *Muribaculaceae*, *Lactobacillus*, bile acid, therapeutic targets

# Introduction

Diabetic kidney disease (DKD) is one of the most common chronic kidney diseases globally, with growing incidence and prevalence (1), as 30% to 40% of patients with diabetes will have complications such as DKD. Chronic inflammation, insulin resistance, poor glycemic control, and oxidative stress have been reported to be driving forces in DKD (2, 3). However, therapies based on these mechanisms have limited effects; more researches about DKD pathogenesis are essential and may provide new insights into treating DKD. Intriguingly, the gut microbiota as a novel intervention for diabetes and its complications, such as DKD, is now attracting more and more attention (4–6).

The gut microbiota has a symbiotic relationship with the host, involving energy metabolism, regulating the gut barrier, and maintaining immune responses (7). Many studies have consistently demonstrated (8–12) that changes in the composition of gut microbiota regulate the development of diabetes by inducing continuous low-grade inflammation and mediating the therapeutic effects of some type 2 diabetes mellitus(T2DM)drugs (13–15).

Sodium glucose co-transporter 2 inhibitors (SGLT2) are the oral treatments for T2DM, with a widely accepted mechanism by reducing the renal threshold of glucose (16). Recently, clinical studies have shown that SGLT2 inhibitors can remarkably prevent DKD progression and the onset of end-stage renal disease independent of lowering glucose (17, 18) and, thus, as SGLT2 inhibitors, Canagliflozin and Dapagliflozin (DAPA) have been used to delay the development of DKD (19). Nevertheless, the underlying mechanisms of SGLT2 inhibitors still need to be fully addressed. For example, some animal studies have shown that SGLT2 inhibitors could reduce albuminuria in db/db mice. While a few recent studies showed SGLT2 inhibitors for 10 weeks did not see any changes in albuminuria using db/db mice, explaining that this phenomenon may be associated with the timing of administration and mild renal histological injury (20, 21). Given that, in this study, we started to administrate DAPA at 6 weeks and set three timepoints at early and late stages of DKD to fully present the renal protective effect of DAPA.

Although Canagliflozin has been reported to reconstruct the gut microbiota in mice with chronic kidney disease (22), there are few and controversial studies about the effects of DAPA on the fecal microbiota of diabetes. Notably, to the best of our knowledge, no study has reported the association between the protection of DAPA on DKD and the gut microbiota. Two studies in 2018 indicated that DAPA could modify the fecal microbiota in animal models of diabetes after 6 or 8 weeks of intervention, accompanied by changing the F/B ratio and microbiota diversity (23, 24). Interestingly, one study in 2020 showed that DAPA did not affect the ratio of F/B and microbiota diversity in a type 2 diabetic rat model at a 1 mg/kg/day dose for 4 weeks (14), implicating the effects of DAPA enhanced possibly over time. Besides, two studies have shown that DAPA, administered for 6 days or 6 weeks, can control blood glucose well without changing colonic or fecal microbiota in the diabetes model, as previous studies described (25, 26). More importantly, one human study reported that DAPA administration did not affect the fecal microbiota in T2DM patients treated with metformin (27). The inconsistency of these results may be related to differences in the length (6 days- 8 weeks) and dose of drug intervention, and the drug combination choice. In short, the DAPA had minor or no effects on the gut microbiota in db/db mice on the condition of the administration period for 6 days - 8 weeks, as most previous studies described. Noteworthily, the administration time of 6 days - 8 weeks is not enough for studying the role of DAPA in DKD associated with its regulation of the gut microbiota. As DAPA is generally continuously used in the long-period treatment of DKD in clinical practice and SGLT2 inhibitors, especially DAPA, are often administrated for 10-12w or longer time rather than 6 days – 8 weeks or less time for the treatment of DKD in db/db mice (21, 28–30); future studies should explore whether DAPA as a novel therapy for DKD can regulate the gut flora and we assumed that the prolonged intervention of DAPA has further benefits. To our knowledge, we firstly suggest that the protective effect of DAPA on DKD may be related to the improvement of the gut microbiota and investigate the impacts of DAPA on the gut flora in the DKD mice over time.

# Methods

## Animals and tissue collection

All animal research was approved by the Institutional Animal Ethics Committee of Renji Hospital. The animal experiment ethics approval number is m20170324. We purchased 5-week-old male C57BL/6 mice and BKS.Cg-Dock7m +/+ Leprdb/J (db/db) mice from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). We housed all mice in a light- and temperature-controlled facility with free access to water and food. After one week of adaptation, we set three groups: control group (C57BL/6 mice administrated with the same volume of physiological saline as the treatment group), model group (db/db mice administrated with the same volume of physiological saline as the treatment group) and treat group (db/db

mice treated with DAPA [1.0 mg/kg/day, AstraZeneca, Cambridge, UK]) respectively at three timepoints of 14 weeks,18 weeks and 22 weeks. DAPA mixed in the drinking water and the same volume of physiological saline were administrated by oral gavage once daily. The body weight and fasting blood glucose levels of the mice were measured every 2 weeks during the treatment period, and the urinary albumin to creatinine ratio (uACR) was measured every 4 weeks. To obtain the pathological gold standard, mice were euthanized at different time points of 8, 12, or 16 weeks following the treatment. Immediately afterwards, the kidneys, intestines, and blood were collected. We pathologically confirmed renoprotective effects of DAPA and then sent the guts of the corresponding mice with DKD improvement to be sequenced.

## Biochemical analysis

We used the Albumin Creatinine Ratio Assay Kit (ab241018) to measure mice's urine albumin concentration and urine creatinine concentration. A Liquid Urea Nitrogen Reagent Set and Creatinine Assay kit (Nanjing Jiancheng Bioengineering Institute, China) was used to measure BUN and plasma creatinine levels.

## Histopathology analyses of renal tissue

We preserved the renal tissues in 10% neutral formalin and embedded them in 10% paraffin. Sections (5 μm thick) were subjected to periodic acid–Schiff (PAS). The glomerulosclerosis index (GSI) was adopted to quantify lesions on PAS-stained paraffin sections. One renal pathologist assessed over 50 glomeruli randomly chosen from each mouse in a blinded manner under ×400 magnification.

## DNA extraction and sequencing

We used the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) to extract total microbial genomic DNA per sample. We used 1.0% agarose gel electrophoresis and a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific Inc., USA) to determine the quality and concentration of DNA, which were kept at -80 °C before the subsequent use. The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified, with all samples amplified in triplicate. We extracted the PCR product from 2% agarose gel and used the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and Quantus™ Fluorometer (Promega, USA) to purify and quantify the PCR product, which was then pooled in equimolar amounts, and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) complied with instructions by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

## Data analysis

We carried out the bioinformatic analysis of the gut microbiota using the Majorbio Cloud platform (https://cloud.majorbio.com). Based on the OTUs information, we calculated alpha diversity indices, including ace richness and Shannon index with Mothur v1.30.1. The similarity among the microbial communities in different samples was determined by β-diversity using the Mothur program.

## Statistical analyses

Data are expressed as mean ± standard deviation (SD). ANOVA was used to evaluate the statistical significance among multiple groups. The statistical significances between the two groups were calculated by Student's unpaired t-test. The significant differences of genera were assessed using Wilcoxon rank-sum test. The differences were considered statistically significant at $P<0.05$. Partial least squares-discriminant analysis (PLS-DA) plots of Bray–Curtis dissimilarity were performed to visualize the group differences.

# Result

## Effects of DAPA on fasting blood glucose and body weight in db/db mice

To evaluate the effects of DAPA on fasting blood glucose and body weight in db/db mice (Figure 1A), one well-known spontaneous diabetic nephropathy model, we measured the changes in fasting blood glucose and body weight over time. The study revealed that db/db mice initially had higher serum glucose than db/m mice. In contrast, a dramatic and consistent decrease in serum glucose was observed in db/db mice after 1 mg/kg DAPA treatment for 8, 12, and 16 weeks (Figure 1B). The mice in the treat group gained weight with survival time. The average body weight at 18 and 22 weeks in the treat group were higher than those in the other groups (Figure 1C), consistent with the result that DAPA could restore weight loss at the late stage of diabetes (23). Notably, the model group did not begin to show a trend of weight loss at 14 weeks (Figure 1C), so it may be reasonable that there were no significant differences in the body weight of mice between the model and treat group at 14 weeks. Collectively, DAPA had a therapeutic effect on hyperglycemia and could significantly reduce weight loss in db/db mice.

## Dapagliflozin effectively slows the progression of DKD in db/db mice

Our results showed that db/db mice had markedly higher uACR levels than db/m mice as expected, the rise of which was a typical manifestation of renal impairment, indicating that early-stage DKD occurred in 6-week-old db/db mice (31). Importantly, DAPA administration at a dose of 1 mg/kg restrained the uACR levels in the treat group at 22 weeks, the late stage of DKD reflected by the remarkable weight loss($P<0.05$) (Figure 2A), interestingly, no significant changes in the uACR levels was seen but with improvements in pathology after DAPA treatment at 14 and 18 weeks in line with one recent study indicating that the uACR level

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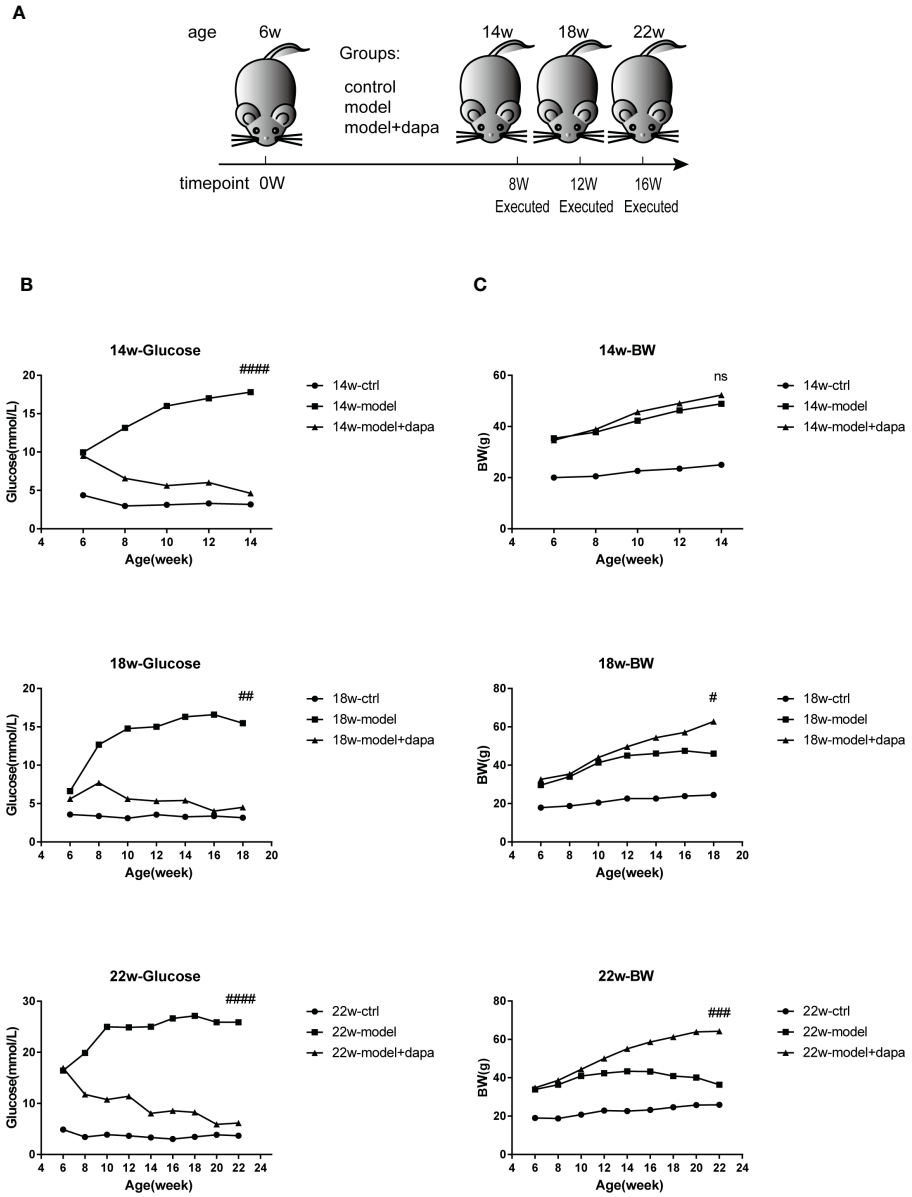


FIGURE 1
DAPA effectively controls fasting blood glucose and body weight in db/db mce. **(A)** the Experimental design for mice. Briefly, six-week-old male nondiabetic db/m and diabetic db/db mice were randomly divided into three group (db/m + physiological saline mice (ctrl group), db/db + physiological saline mice (model group), and db/db model mice + DAPA (model +DAPA group)) and administrated by oral gavage once daily with physiological saline or 1.0 mg/kg/day DAPA, finally executed respectively at 14w (14 weeks), 18w (18 weeks), and 22w(22 weeks). **(B)** Fasted blood glucose levels respectively from 6w to 14w, 18w, and 22w. **(C)** Changes in body weight respectively from 6w to 14w, 18w, and 22w. Statistical significance was calculated using ANOVA with Tukey's test. N=4/group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$, ns, not significant for model group vs model + DAPA group at the same time point. ctrl: the control group.

changes can be seen with more severe renal histological injury in the late stage of DKD. No significant differences were observed in serum creatinine and urea nitrogen levels among the three groups during the observation period (Figures 2B, C). Periodic acid-Schiff staining of renal tissue showed that db/db mice in the model group exhibited increased renal structure damage, such as glomerular mesangial matrix expansion and mesangial hyperplasia compared to the control group; the pathologies mentioned above were remarkably alleviated in the treatment group compared to the model group at the same time point of 14, 18, 22 weeks (Figures 2D, E), indicating that DAPA treatment successfully protected from kidney damage.

## Dapagliflozin gradually modulates the overall structure of the gut microbiota in db/db mice

To compare the α-diversity and β-diversity of the gut microbiota composition among the three groups at three timepoints, we subjected fecal samples of three groups at different timepoints to 16S rRNA analysis. Bacterial community diversity was measured by the Shannon index and ace index, which indicates bacterial community richness. The Shannon index of the DAPA-treated group was significantly lower than that of the model group (Figure 3A); however, the ace index of the treat

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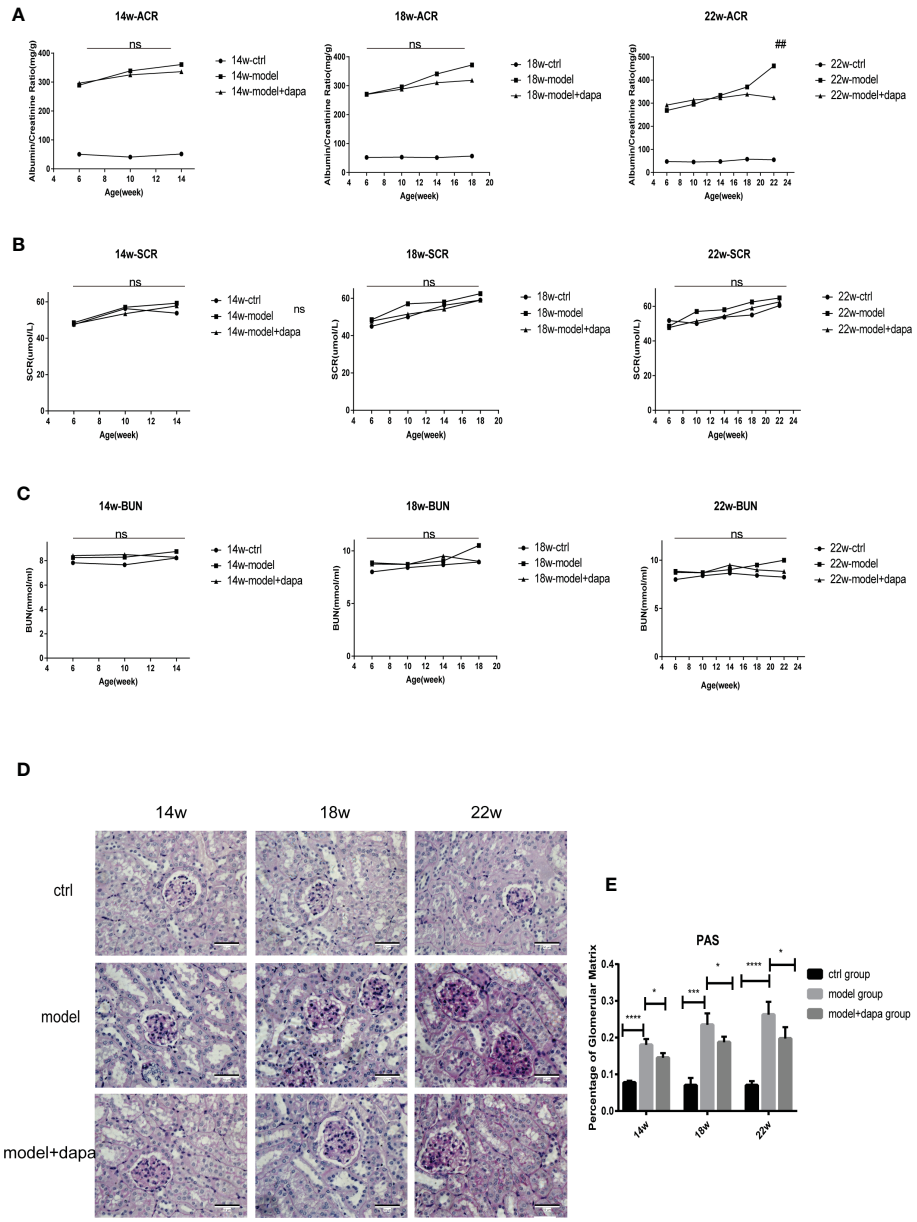


FIGURE 2
DAPA effectively slows the progression of DKD in db/db mice. **(A–C)** Albumin to creatinine ratio (ACR), scr, and bun levels were determined every 4 weeks in the mice of three groups throughout 14 weeks (14w), 18 weeks (18w), and 22 weeks (22w). **(D, E)** Periodic acid-Schiff (PAS) staining analysis of the histopathological changes among three groups at 14w, 18w, and 22w. original magnification, x400. Scale bars, 50 μm. Data in **(D)** were quantified **(E)**. n = 4/group. Statistical significance was calculated using ANOVA with Tukey's test. *p < 0.05, ***p < 0.001, ****p < 0.0001. ns, not significant for the indicated comparison. Ns in **(A)** is for comparison between the model group and model + DAPA group, ns in **(B, C)** is for comparison among three groups. **p < 0.01 for model group vs mode; + DAPA group.

group had an increasing but not significant trend compared with the control group at 14 weeks (p=0.056) (Figure 3B). As we expected, the Shannon index was found to be higher in the treat group compared to the model group at 18 weeks with the therapy time prolonged (Figure 3C), although the ace index did not differ between the two groups (Figure 3D). At 22 weeks, there were increasing but not significant trends in the Shannon index and ace index after DAPA treatment (Figures 3E, F), which may result from the variances in the degree of disease development in mice within groups. Besides, although the indexes of richness and diversity failed to show a notable change between the model group and the control group at 14, 18, and 22 weeks, partial least squares-discriminant analysis (PLS-DA) plots of Bray–Curtis dissimilarity at three timepoints showed that the dots of the model group were not close to the dots of the control group, indicating there are distinct differences in the structure of intestinal flora between the two groups (Figures 3G–I). Remarkably, although the PLS-DA at 14 weeks suggested that the gut microbiota composition of the three groups was far apart, the plots of the treat group were closer to the fields of the control group than the model group to the control group at 18 weeks and 22 weeks (Figures 3G–I), which may be driven by a more extended intervention of DAPA. The heat maps of Bray–Curtis distance presented similar findings (Supplementary Figures 1A–C). Taken together, these results suggested that DAPA could dramatically alter the structure of the gut microbiota in a time-dependent manner.

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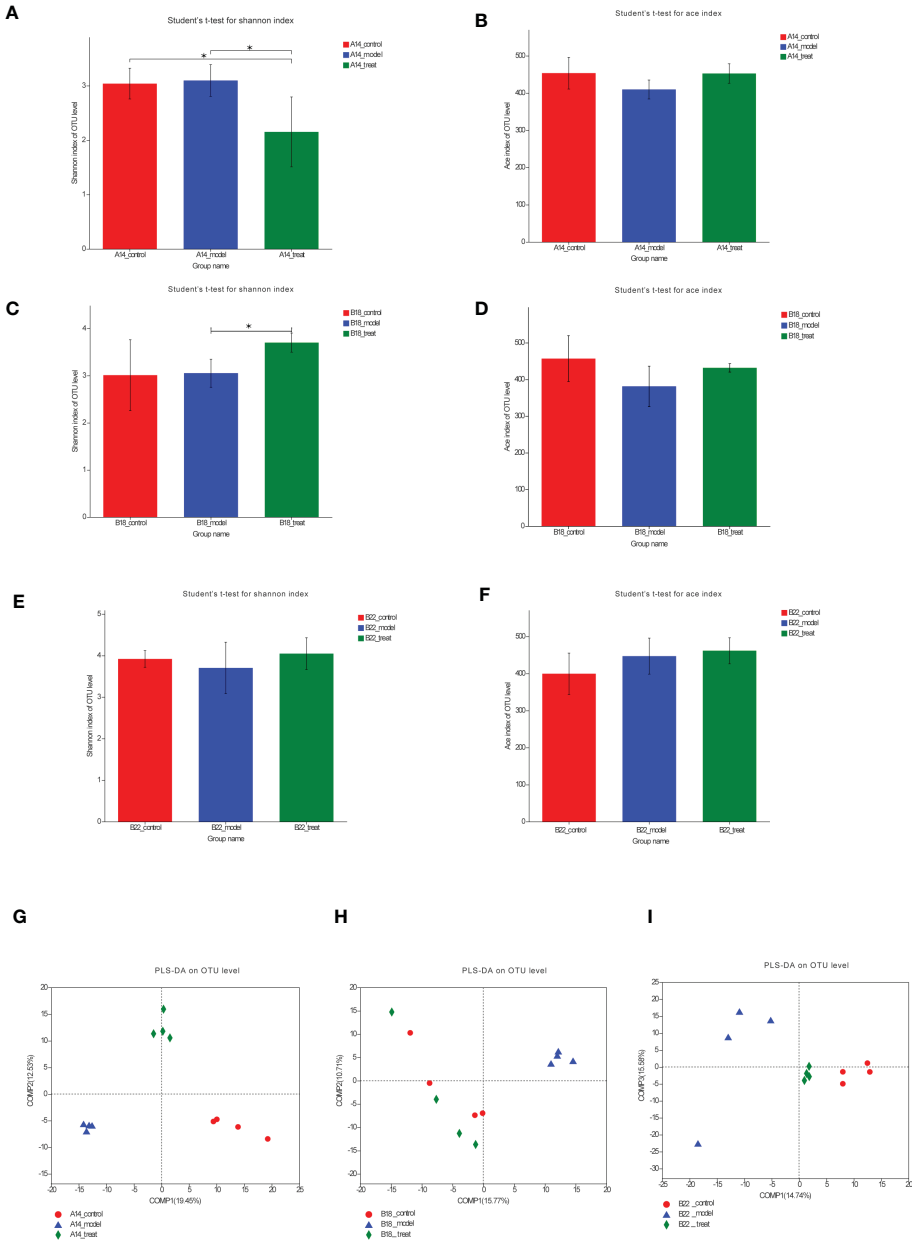


FIGURE 3
DAPA gradually modulates the overall structure of the gut microbiota in db/db mice. **(A, C, E)** Shannon diversity and ace richness **(B, D, F)** of fecal samples across three groups OUT at 14 weeks (14w), 18 weeks (18w), and 22 weeks (22w). **(G–I)** PLS-DA of OTU-level Bray-Curtis at 14w, 18w, and 22w. n = 4/group. Data are expressed as mean ± SEM. Statistical significance was calculated using ANOVA with Tukey's test. *p < 0.05. PLS-DA, partial least squares-discriminant analysis. The treat group means model + DAPA group.

## The effect of dapagliflozin to dramatically restore the dysbiosis of db/db mice at the phylum levels enhanced over time

As shown in Figure 4A, although Firmicutes and Bacteroides still accounted for the most significant proportion in the overall structure of intestinal flora from three groups at different time points, the relative abundance of some bacteria changed a lot. Specifically, compared to the control group, the model group had a higher quantity of Proteobacteria and a relatively lower abundance of Patescibacteria at 14 weeks (Figure 4B), while at 18 weeks, the relative abundance of Verrucomicrobiota decreased (Figure 4C), accompanied by an increased but not significant trend of Firmicutes. As the molding time lengthens, the model group was characterized by remarkably elevated levels of Firmicutes together with decreased abundance of Bacteroidetes compared to the control group at 22 weeks (Figure 4D), indicating the gut microbiota of db/db mice being gradually disordered over time and Bacteroidetes and Firmicutes may have crucial impacts on the DKD during the disease progression.

Further comparison of the bacterial taxa revealed differences between the treat and model groups. The treat group showed a lower abundance of Desulfobacterota at 14 weeks (Figure 4E). Interestingly, DAPA treatment increased the relative abundance of Bacteroidota at 18

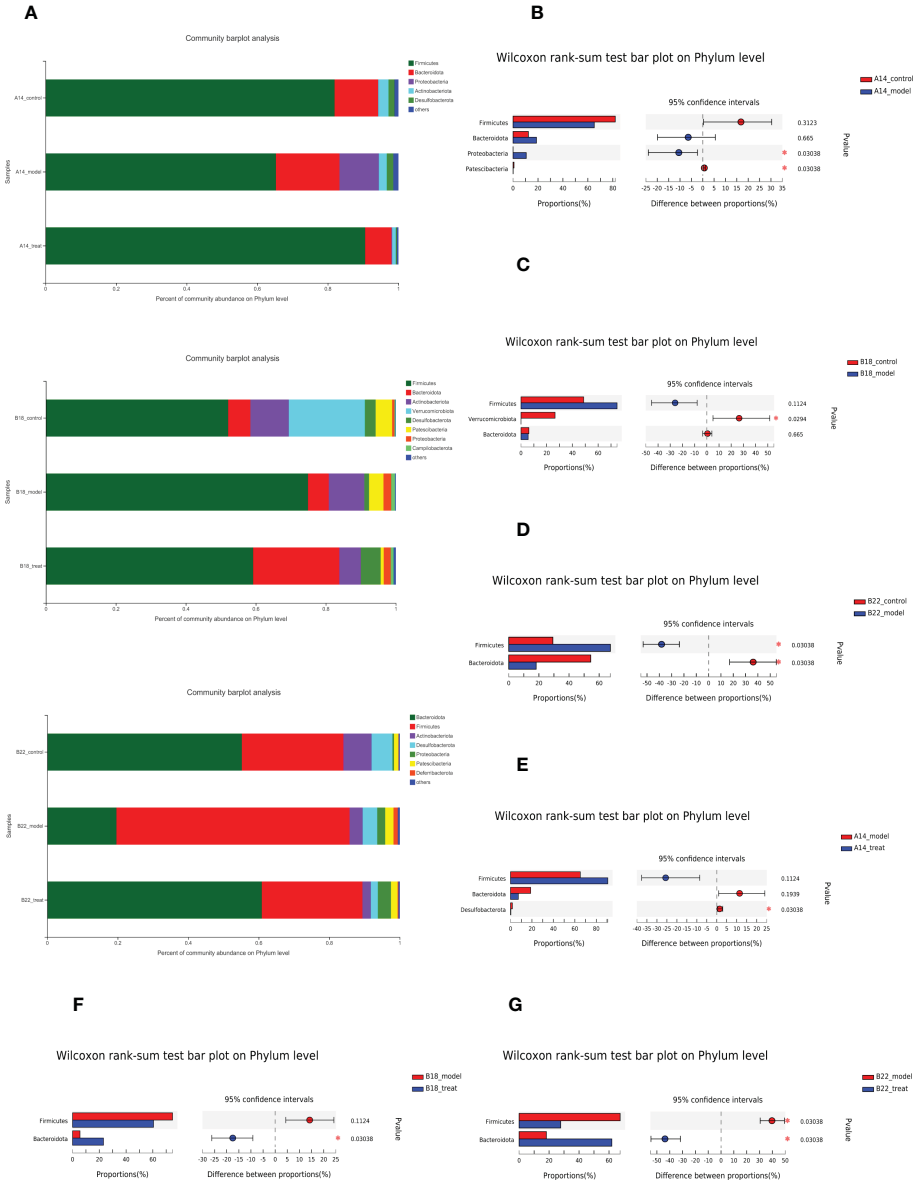


FIGURE 4
The effect of DAPA to dramatically restore the dysbiosis of db/db mice at the phylum levels enhanced over time. **(A)** Relative abundance of bacterial phyla, indicating changes in microbiota composition among three groups at 14 weeks (14w), 18 weeks (18w), and 22 weeks (22w). **(B–D)** Presentation of phyla with significant changes between the control group and model group at 14w, 18w, and 22w apart from dominant Firmicutes and Bacteroidota. **(E–G)** Presentation of phyla with significant changes between model group and model + DAPA group at 14w, 18w, and 22w besides dominant Firmicutes and Bacteroidota. N=4/group. Statistical significance was calculated using Wilcoxon rank-sum test. *p < 0.05. The treat group means model + DAPA group.

weeks (Figure 4F) and then consistently rescued flora disorder of DKD by reducing the relative abundance of Firmicutes and further increasing the relative abundance of Bacteroidota at 22 weeks (Figure 4G), which strongly suggested that the effect of DAPA on the gut microbiota of db/db mice enhanced with time.

## Dapagliflozin consistently remodels the gut microbiota composition of db/db mice at the genus level

To further investigate the changes in the microbiota signature among groups, the analysis was carried out at the genus level (Figures 5A–F and Supplementary Figures 2A–C). Compared with the control group, *Escherichia-Shigella*, *Enterococcus*, *Citrobacter*, etc., were strikingly elevated. In contrast, *Roseburia*, *unclassified_f: Lachnospiraceae*, *Alistipes*, etc., in the DKD model group were markedly reduced at 14 weeks (Figure 5A). Interestingly, the results showed that the expansion of detrimental intestinal bacteria at 14 weeks was restrained after DAPA treatment. Specifically, *Escherichia-Shigella*, *Enterococcus*, *norank_f:Desulfovibrionaceae*, *Eubacterium_nodatum_group*, etc., decreased. Besides, *Lachnospiraceae_NK4A136_group*, *Colidextribacter*, *unclassified_f: Oscillospiraceae*, *Blautia*, *Odoribacter*, *etc.* increased in the treat group compared with the model group at 14 weeks (Figure 5B). With the disease progression, at 18 weeks, in addition to *Escherichia-*

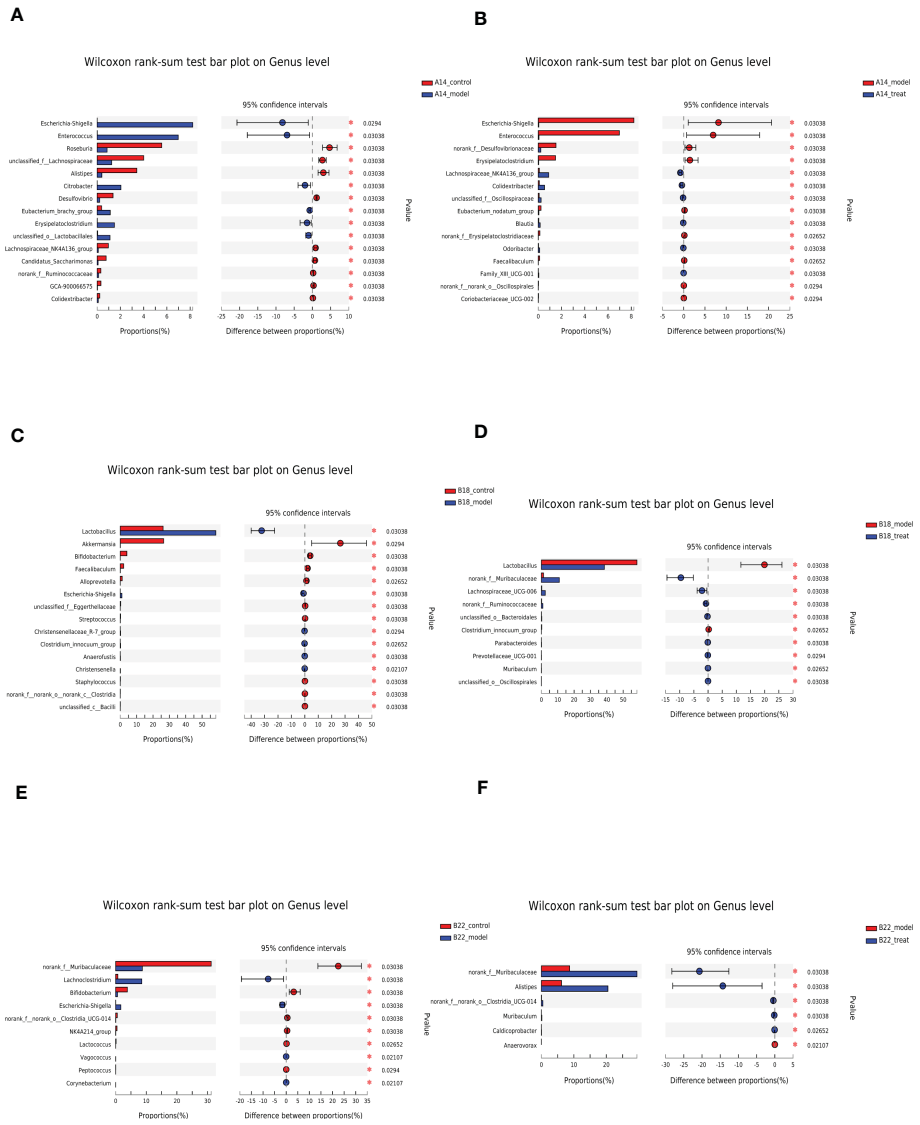


FIGURE 5
DAPA consistently remodels the gut microbiota composition of db/db mice at the genus level. **(A, C, E)** Significantly changed top 15 genera sorted by their abundances between the control group and model group at 14 weeks (14w), 18 weeks (18w), and 22 weeks (22w). **(B, D, F)** Significantly changed top 15 genera sorted by their abundances between the model group and model + DAPA group at 14w, 18w, and 22w. n=4/group. Statistical significance was calculated using Wilcoxon rank-sum test. *p < 0.05. The treat group means model + DAPA group.

*Shigella*, *Lactobacillus* also significantly increased in the model group compared to the control group (Figure 5C), which was strikingly restored by DAPA administration (Figure 5D). Besides, beneficial bacteria such as *Akkermansia*, *Bifidobacterium*, *Faecalibaculum*, *Alloprevotella*, etc., in the model group decreased compared with the control group (Figure 5C). Notably, DAPA administration greatly enriched beneficial *norank_f:Muribaculaceae* apart from *Lachnospiraceae_UCG-006*, *norank_f:Ruminococcaceae*, *unclassified_o:Bacteroidales*, *Parabacteroides*, *Prevotellaceae_UCG-001*, *Muribaculum*, *unclassified_o:Oscillospirales*, etc. compared with the model group (Figure 5D).

At 22 weeks, in the model group, *Escherichia-Shigella* and *Lachnoclostridium* increased along with *Lactobacillus* having an increasing tendency (Figure 5E). On the other hand, *norank_f:Muribaculaceae*, *Bifidobacterium*, *norank_f:norank_o:Clostridia_UCG-014*, *NK4A214_group*, and *Lactococcus* decreased compared with the control group (Figure 5E). Greatly decreased *norank_f:Muribaculaceae* resulting from DKD can be rescued by DAPA administration which also boosted *Alistipes*, *norank_f:norank_o:Clostridia_UCG-014*, *Muribaculum*, *Caldicoprobacter*, etc. and reduced *Anaerovorax*. Notably, the expansion of *Lactobacillus* was restricted after DAPA administration, although there is no significant difference compared with the model group (Figure 5F and Supplementary Figure 2C).

## Bile acid may be associated with CKD progression and one of the DAPA intervention target

No significant KEGG pathway was enriched using PICRUSt analysis for the fecal microbiome among three groups at 14 weeks,

which may be due to the early stage of DKD and short DAPA intervention (Supplementary Figure 3A). However, the pathway of primary bile acid biosynthesis was significantly upregulated in the DKD group at 18 weeks and *bilophila* tended to rise in the DKD group at 22 weeks (Supplementary Figures 3B, C). DAPA intervention tended to reverse the bile acid change, although it was not significant (Supplementary Figures 3B, C).

Overall, these results suggested that DAPA increasingly and consistently prevented DKD-driven dysbiosis at the genus level, phylum level and overall structure of the gut microbiota, which may be associated with the effect of DAPA on bile acid pool and its antioxidation effect.

## Discussion

We presented evidence that daily administration of db/db mice with DAPA was sufficient to prevent diabetes-induced weight loss and hyperglycemia, further easing the DKD, indicated by strikingly reduced proteinuria and deposition of mesangial matrix showed by Periodic acid-Schiff staining. Although studies showed that DAPA could reduce body weight, we observed that the weight of the treat group gradually increased, while the db/db mice had different degrees of weight loss at 18 weeks and 22 weeks, which are consistent with the previous research (23, 26, 32). In this regard, weight gains after DAPA intervention may reflect the improvement in disease status in db/db mice.

To explore the characterization of dysbiosis implicated in the progression of DKD and further determine whether DAPA administration could restore the structure of the gut microbiota or not, we performed 16s rRNA sequencing of fecal samples.

Regarding the first purpose, we firstly showed the increasing gut microbiota disorder and bile acid in DKD. Specifically, at the phylum level, the results at 14 weeks showed that the abundance of Proteobacteria, containing many harmful bacteria, was highly elevated, accompanied by diminished beneficial Patescibacteria without changing Firmicutes and Bacteroidota, while at 18 weeks, the probiotic Verrucomicrobiota widely spread in the healthy human intestine suppressing the inflammation process declined and the Firmicutes has an increasing but not significant trend. Interestingly, as the DKD progressed, a further increase in the proportion of Firmicutes occurred along with a decrease in Bacteroidota at 22 weeks which is common in diabetes and cardiac diseases. Besides, this result was consistent with a previous study showing that the increased F/B ratio was not always linked with a fat phenotype (23). Likewise, similar alterations were observed at the genus level. In the model group, conditional pathogenic bacteria such as *Escherichia-Shigella* expanded; however, beneficial bacteria such as *Roseburia*, *unclassified_f:Lachnospiraceae*, *Alistipes*, *Akkermansia*, and *Bifidobacterium* diminished. The *Lactobacillus* belonging to the Firmicutes has an increasing trend, and the *norank_f:Muribaculaceae* belonging to the Bacteroidetes showed a decreasing trend, which is more evident at 22 weeks, namely the late stage of the disease. Taken together, this trend at different levels was more pronounced at 22 weeks and 18 weeks than at 14 weeks consistent with KEGG pathways enrichment results. Therefore, there was a developing dysbiosis and bile acid accumulation in the progression of DKD. Our study raised the possibility that regulating the gut microbiota could be a promising strategy for DKD therapy. Based on that, it is reasonable to hypothesize that the effect of DAPA on the gut microbiota could mediate its protective role.

More importantly, consistent with the second hypothesis, our study showed that DAPA influenced the composition of intestinal flora, and this effect was enhanced with the prolongation of intervention time. On the whole, the α diversity was reduced in the treat group compared with the model group at 14 weeks; although this did not meet our expectations, but this is consistent with a previous study in which microbial diversity declined after the same short period of DAPA administration for improving diabetes and vascular dysfunction (23). Interestingly, with the intervention going on, α diversity increased significantly at 18 weeks and also showed an increasing trend at 22 weeks. Besides, the PLS-DA results further demonstrated the protective effect of DAPA on the gut microbiota over time. At the phylum level, the initial DAPA response at 14 weeks appeared to be driven by minor differences across phyla rather than noticeable changes of Firmicutes and Bacteroidetes due to short intervention explaining no significant KEGG pathways enrichment, and later the response at 18 weeks and 22 weeks were driven by significant changes of certain bacteria from the two predominant phyla. In our study, after DAPA treatment for 16 weeks, Bacteroidetes increased, and Firmicutes decreased at 22 weeks, suggesting DAPA rescued the overall changes in the gut microbiota of DKD. Bacteroidetes and Firmicutes take on responsibilities in improving glucose metabolism and lipid metabolism with enzymes such as α-glucosidases and α-amylases and hold the balance on gut microbiota due to their large proportion (33). Moreover, the bigger F/B ratio in the gut flora was reported to be associated with more pro-inflammatory cytokines and stronger insulin resistance (34, 35). Therefore, the regulation of DAPA on Bacteroidetes and Firmicutes might be necessary for its hypoglycemic effects.

The effects of DAPA on the gut flora at the genus level were consistent with the whole. At 14 weeks, DAPA did not enrich beneficial bacteria such as *Bifidobacteriaceae* but confined the boom of pathobionts, including *Escherichia-Shigella* and *Enterococcus*, related to impairing the intestinal barrier and therefore worsening kidney disease by activating the innate immune system in line with the previous study with the same short intervention (14). Other protective *Lachnospiraceae_NK4A136_group*, *Colidextribacter* abundant in healthy controls (36), *unclassified_f:Oscillospiraceae*, *Blautia producing acetic acid* (37) and *Odoribacter* associated with succinate consumption (8) expanded.

At both 18 and 22 weeks, DAPA consistently reversed the abundance changes of *Lactobacillus* and *norank_f:Muribaculaceae*, reflecting the principal change of firmicutes and Bacteroides in DKD mice. In agreement with previous studies (10, 35), the percentage of *Lactobacillus* increased in the diabetic model. Our study showed that DAPA could reverse the trend, strongly implicating that *Lactobacillus* may play a role in metabolic disorders emerging in the progression of DKD as *Lactobacillus* was recently reported to induce or maintain low-grade inflammation (10, 14, 38, 39). *Muribaculaceae*, also named the S24-7 and belonging to Bacteroidetes, was the dominant bacterium at 18 and 22 weeks in treat group, in line with previous

studies (13, 14). The abundance of *Muribaculaceae* has been implicated in predicting the levels of short-chain fatty acids (SCFAs), such as propionate production, and partially mediated the exercise-driven prevention of obesity (40–43). Other DAPA-enriched genera also have potential benefits. For example, *Alistipes* is a genus of Bacteroidetes mainly producing SCFAs such as acetate and propionate (44). Besides, *Ruminococcus* can inhibit the production of ROS by making ursodeoxycholic acid (45). Taken together, these results indicated that DAPA could remodel the gut microbiota to increase the production of SCFAs and perform the anti-inflammatory property, therefore ameliorating kidney damage.

Notably, the abundance of *Lactobacillus* seemed to be always negatively associated with the quantity of *norank_f:Muribaculaceae* in the DKD model group and DAPA intervention group. Thinking about the association between Firmicutes and Bacteroides at 18 weeks and 22 weeks, by searching PubMed online articles, one recent report in 2022 concluded that acids produced by *lactobacilli* inhibited the growth of commensal *Lachnospiraceae* and *Muribaculaceae* (46). Besides, similar to the microbial alteration pattern of negative correlations in our study, studies showed that treatment with intervention hugely reversed the increased F/B ratio, which is the hallmark of dysbiosis, accompanied by a striking downregulation of *Lactobacillus* belonging to Firmicutes (47, 48). One possible explanation for the phenomenon is that bile acid favored genus belonging to Firmicutes, such as BA (bile acid)-resistant *Lactobacillus* (35), and reducing the bile acid pool may remove the competitive advantage of *Lactobacillus spp* (47–49). Interestingly, our study also showed increased bile acid in the later stage of DKD reflected by the enriched primary bile acid biosynthesis at 18 weeks and increased *bilophila* at 22 weeks in the model group, at least partly explaining the expanded Firmicutes in the model group in our study. Besides, DAPA intervention in our study tended to reverse the bile acid change, possibly resulting in the reversed F/B ratio. Moreover, the antioxidant property of specific intervention may restrain oxygen availability and further compromise the bloom of facultative anaerobe species such as *Lactobacilli* (47, 50). Indeed, the disorder of bile acid metabolism exists in DKD patients (10, 51). The change in bile acids induced by medication has also been reported to be correlated with better clinical outcomes, thereby improving the metabolic health of DKD (52, 53). In addition, DAPA could function by reducing the generation of ROS (54). Therefore, DAPA-driven modulation in Bacteroidetes and Firmicutes may root in the impacts of DAPA on the bile acid pool and its antioxidation effect. However, whether DAPA delayed the progression of DKD *via* regulating the gut microbiota such as *norank_f:Muribaculaceae* and *Lactobacillus* and especially bile acid, needs to be further verified in future research.

In conclusion, DAPA remarkably protected the progression of DKD. Its impact on modulating the flora of db/db mice at 14 weeks was roughly in line with the previous study, and our study uniquely presented its increased effects reflected by the consistent changes at phylum and genus levels, which may be associated with its regulation on bile acid at 18 and 22 weeks. To the best of our knowledge, we firstly suggest that the protective effect of DAPA on DKD may be related to the improvement of the gut microbiota, possibly linked with bile acid in a time-dependent manner, which provides more solid evidence that prolonged DAPA intervention enhances the regulation of dysbacteriosis in DKD and renders a new target for DKD therapy.

## Data availability statement

The data presented in the study are deposited in the SRA database, accession number PRJNA923132.

## Ethics statement

The animal study was reviewed and approved by the Institutional Animal Ethics Committee of Renji Hospital.

## Author contributions

JW and YC designed the experiments, analyzed the data, and wrote the manuscript. HY carried out the experiments. LG and ZN provided critical materials. SM revised the manuscript. XC and JS supervised the project. All authors have read and approved the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1026040/full#supplementary-material

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# Probiotics intervention in preventing conversion of impaired glucose tolerance to diabetes: The PPDP follow-on study

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**Objectives:** The purpose of this study was to assess the incidence of type 2 diabetes mellitus (T2DM) after 6 years in patients with IGT who received early probiotic intervention in the Probiotics Prevention Diabetes Program (PPDP) trial.

**Methods:** 77 patients with IGT in the PPDP trial were randomized to either probiotic or placebo. After the completion of the trial, 39 non-T2DM patients were invited to follow up glucose metabolism after the next 4 years. The incidence of T2DM in each group was assessed using Kaplan-Meier analysis. The 16S rDNA sequencing technology was used to analyze gut microbiota's structural composition and abundance changes between the groups.

**Results:** The cumulative incidence of T2DM was 59.1% with probiotic treatment versus 54.5% with placebo within 6 years, there was no significant difference in the risk of developing T2DM between the two groups ($P$=0.674).

**Conclusions:** Supplemental probiotic therapy does not reduce the risk of IGT conversion to T2DM.

**Clinical Trial Registration:** https://www.chictr.org.cn/showproj.aspx?proj=5543, identifier ChiCTR-TRC-13004024.

KEYWORDS

probiotic, impaired glucose tolerance, diabetes mellitus, type 2, gut microbiota, conversion

# 1 Introduction

Compared with normal glucose tolerance (NGT), people with prediabetes, especially impaired glucose tolerance (IGT), have a higher risk of developing type 2 diabetes mellitus (T2DM). Early intervention can significantly reduce the probability of developing T2DM in the IGT population (1–3). The dysbiosis in the gut microbiota has recently been recognized as a critical environmental factor in individuals with prediabetes or diabetes mellitus (DM) (4, 5). Several studies (6–8) showed that probiotic and synbiotic intake affects the glycemic profile in patients with prediabetes and T2DM. Most recently, the Probiotics Prevention Diabetes Program (PPDP) Study demonstrated that probiotic supplementation during two years did not improve fasting plasma glucose(FPG) levels and did not reduce the risk of conversion of IGT to T2DM (9).

After the PPDP trial was completed, participants without T2DM were invited to follow up for 4 years. The objective of the PPDP Follow-On study was to observe the effect of early probiotic intervention on the conversion of T2DM after 6 years.

# 2 Research design and methods

## 2.1 PPDP study

The design and primary results of the PPDP study have been reported previously (9, 10). Briefly, the PPDP Study included 77 patients diagnosed with IGT in the outpatient department of Shanghai East Hospital of Tongji University from September 2014 to September 2016. IGT and T2DM were diagnosed according to the 1999 WHO Criteria. IGT was diagnosed when FPG <7.0 mmol/L, and oral glucose tolerance test (OGTT): 2-h post glucose load ≥ 7.8 and <11.0 mmol/L. T2DM was diagnosed when FPG ≥7.0 mmol/L and or OGTT: 2-h post glucose load≥11.1 mmol/L, or self-reported diabetes history and being treated with hypoglycemic agents. All participants were randomized, double-blind to receive probiotics (including Bifidobacterium, Lactobacillus acidophilus and Enterococcus faecalis) or matched placebo. Probiotics were produced by Shanghai Sine Pharmaceutical Laboratories Co, Ltd. For both groups, the doses were 840 mg daily, 210 mg per one pill, two pills per time, and two times daily. Both groups were followed for two years with an OGTT every 3 months in the first year and every 4 months in the second year to assess the patient's glucose metabolism. At the end of the PPDP study, there were 20 patients in the Probiotics group and 13 in the Placebo group who developed T2DM.

Feces of the two groups before and after intervention were collected. The 16S rDNA sequencing technology was used to analyze intestinal microbiota's structural composition and abundance changes. The primary outcome was the cumulative prevalence of T2DM in the two groups. The secondary endpoints were the possible changes in the proportion of microbiota. The study was registered in the Chinese clinical trial registry (ChiCTR-TRC-13004024).

## 2.2 PPDP follow-on study

After the completion of the initial PPDP study, patients who with undiagnosed T2DM continue to be invited to participate in the PPDP Follow-On study without probiotics intervention. A total of 39 non-T2DM patients agreed to follow up glucose metabolism for next 4 years. Patients were asked to monitor fasting and postprandial blood glucose by themselves. At the 4th year, OGTT were assessed at the outpatient department of Shanghai East Hospital. Finally, 36 patients finished the next 4-year follow-up, 2 patients withdrew due to loss of contact, and 1 patient died due to a blood tumor, with a dropout rate of 4.2%. The detailed patient flow of the original trial (PPDP) and follow-on study is summarized in Figure 1.

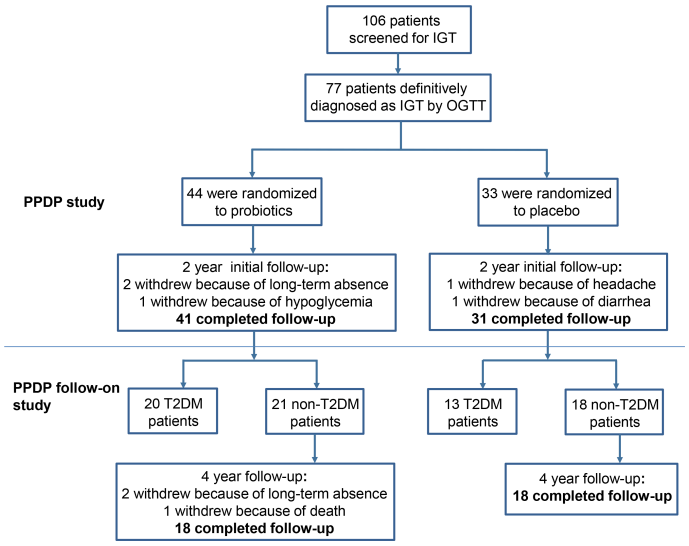


FIGURE 1
Flow diagram for PPDP follow-on study.

The primary outcome was the cumulative incidence of T2DM in the two groups during the 6 years. The PPDP study and the PPDP Follow-On study were reviewed and approved by the hospital's ethics committee and all patients signed informed consent.

## 2.3 16S rRNA gene sequencing and analysis

Fresh fecal samples were collected and bacteria's 16S rRNA gene sequence was detected using paired-end configuration on an Illumina MiSeq system (Illumina, San Diego, USA). Briefly, microbial DNA was extracted and DNA quality was examined by agarose gel electrophoresis. The V3-V4 regions of the bacteria's 16S rRNA gene were amplified by PCR. The sequencing was performed using paired-end configuration on an Illumina MiSeq system (Illumina, San Diego, USA). Raw fastq files were demultiplexed, and then data was filtered to ensure quality. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the silva (SSU115)16S rRNA database. The detail of sequencing and analysis showed in Supplementary Material 1.

## 2.4 Statistical analyses

Statistical analyses were performed by SPSS version 23.0 (IBM Corp, Armonk, NY, USA) and GraphPad Prism version 8.0 (San Diego, California, USA). Continuous data were described as means ± standard deviation, and inter-group comparison was performed with a t-test or analysis of variance. All continuous data were abnormally distributed. Categorical data were described as n (%), and inter-group comparisons were analyzed by $\chi^2$ -test. Mann–Whitney test was used to compare data that were not normally distributed between the groups. The cumulative incidence of T2DM = (the number of cases developing T2DM after 6 years of follow-up/ the number of cases starting follow-up) × 100%. The difference in the incidence of T2DM between the probiotic group and the placebo group over time was analyzed using the Kaplan-Meier survival curve. COX regression analysis was used to analyze the influencing factors of T2DM. The risk was described as Hazard ratio (HR) and 95%CI. P<0.05 was statistically significant.

# 3 Results

## 3.1 Baseline characteristics

The baseline characteristics of the Probiotics group and Placebo group in the PPDP study have been presented in the previous article (9). Specifically, there were no significant differences in sex composition, age, body mass index (BMI), blood pressure, heart rate, liver function, blood lipid profile, FPG, post-glucose load plasma glucose, glycated hemoglobin A1c(HbA1c), fasting serum insulin (FINS) and the homeostasis model assessment of insulin resistance (HOMA-IR) between the two groups.

At the end of the PPDP study, there remained 39 patients with undiagnosed T2DM (21 patients in the Probiotics group and 18 in the Placebo group). The characteristics of undiagnosed T2DM patients at the end of 2-year follow up between the probiotics and placebo groups were shown in Table 1.

## 3.2 Comparison of the incidence of T2DM

In the next 4 years, there were 6 patients in the Probiotics group and 5 patients in the Placebo group who developed T2DM. Thus, the cumulative incidence of T2DM was 59.1% in the probiotic group and 54.5% in the placebo group within 6 years. As shown in Figure 2, there was no significant difference in the risk of developing T2DM between the two groups within 6 years ($P$=0.674).

At the end of the 6-year follow-up, patients were grouped according to whether T2DM occurred. The age of the T2DM group was significantly older than the non-T2DM group (57.2 vs 53.7 years, $P$=0.004). There was no significant difference in other clinical data between the two groups (all $P$ >0.05).

## 3.3 COX regression analysis of risk factors for T2DM

COX regression model was used to analyze the risk factors affecting the development of T2DM. Probiotic intervention or not, age, gender, BMI, waist circumference, blood pressure, liver function, blood lipid, blood glucose, serum insulin and HbA1c were used as covariates, and results showed that 30-minute post-glucose load insulin level was a factor affecting the conversion of IGT to T2DM (HR=0.954, 95%CI 0.915-0.994, $P$ =0.026) (Figure 3).

## 3.4 Gut microbiota analysis

According to the informed consent and research protocol, fecal samples were collected at baseline (day 0) and the end of the 2-year follow-up visit. The 16S rDNA sequencing technology was used to analyze gut microbiota's structural composition and abundance changes. A total of 32 stool samples in the Probiotic group and 22 in the Placebo group were collected. In this study, the differences in operational taxonomic units (OTUs) abundance among the probiotic group and the placebo group were compared. The Venn diagram showed that 435 of the total 972 genera were shared among the 4 groups (Figure 4A). To display microbiome space between samples, principal coordinates analysis (PCoA) was performed. The results showed that the microbiota was similar between the probiotic and placebo interventions (Figure 4B), and the probiotic intervention might not have caused the recombination of the microbial community composition. Microbial community variation was also analyzed. At the genus level, Blautia, Subdoligranulum, Eubacterium hallii, Bifidobacterium, and Romboutsia accounted for the majority in each group (Figure 4C). However, there were no statistically significant changes in the microbiota composition after probiotics or

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TABLE 1 Characteristics of undiagnosed T2DM patients at the end of 2-year follow up between the Probiotics and Placebo groups.

| | Probiotics group (n=21) | Placebo group (n=18) | *P* value |
|---|---|---|---|
| Age (year) | 62.3 ± 10.2 | 52.1 ± 14.7 | 0.015* |
| Male n(%) | 7 (33.3) | 9 (50.0) | 0.342 |
| BMI (kg/m$^2$) | 25.2 ± 2.3 | 24.3 ± 2.3 | 0.483 |
| WC (cm) | 88.2 ± 7.5 | 87.8 ± 11.9 | 0.903 |
| SBP (mmHg) | 125.3 ± 12.3 | 119.3 ± 18.3 | 0.270 |
| DBP (mmHg) | 79.1 ± 9.4 | 76.1 ± 8.7 | 0.348 |
| ALT (IU/L) | 24.8 ± 15.8 | 23.4 ± 23.3 | 0.851 |
| SCr (umol/L) | 64.8 ± 9.4 | 62.1 ± 12.0 | 0.690 |
| TG (mmol/L) | 1.2 ± 0.59 | 1.69 ± 1.09 | 0.181 |
| TC (mmol/L) | 4.51 ± 0.99 | 4.99 ± 1.01 | 0.157 |
| HDL-C(mmol/L) | 1.51 ± 0.30 | 1.43 ± 0.48 | 0.555 |
| LDL-C (mmol/L) | 2.91 ± 0.91 | 3.22 ± 0.97 | 0.212 |
| FPG (mmol/L) | 5.25 ± 0.47 | 5.23 ± 9.54 | 0.933 |
| 30minPG (mmol/L) | 9.72 ± 1.62 | 9.40 ± 1.79 | 0.604 |
| 2hPG (mmol/L) | 7.76 ± 1.25 | 7.23 ± 0.88 | 0.154 |
| FINS (μU/L) | 11.7 ± 7.9 | 11.1 ± 10.8 | 0.850 |
| 30minINS (μU/L) | 77.8 ± 53.8 | 75.5 ± 61.1 | 0.906 |
| 2hINS (μU/L) | 99.2 ± 64.4 | 64.4 ± 47.4 | 0.075 |
| HOMA-IR | 2.79 ± 2.07 | 2.59 ± 2.45 | 0.802 |
| Incidence of T2DM 4 years later (n) | 6 | 5 | – |

ALT, Alanine aminotransferase; BMI, body mass index; DBP, Diastolic blood pressure; FPG, fasting plasma glucose; FINS, fasting insulin; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, Triglyceride; SCr, serum creatinine; 30minPG, 30-minute-plasma glucose post-glucose load; 2hPG, 2-h-plasma glucose post-glucose load; 30mINS, 30-minute-insulin post-glucose load; 2hINS, 2-hour-insulin post-glucose load. Categorical data were described as n (%). Continuous data are presented as mean ± SD.
*P values<0.05 were considered significant.

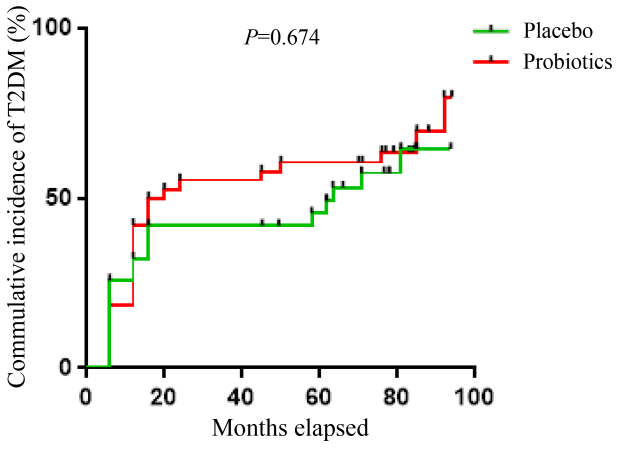


FIGURE 2
Kaplan-Meier analysis of the cumulative incidence of T2DM within 6 years between the probiotics and placebo group.

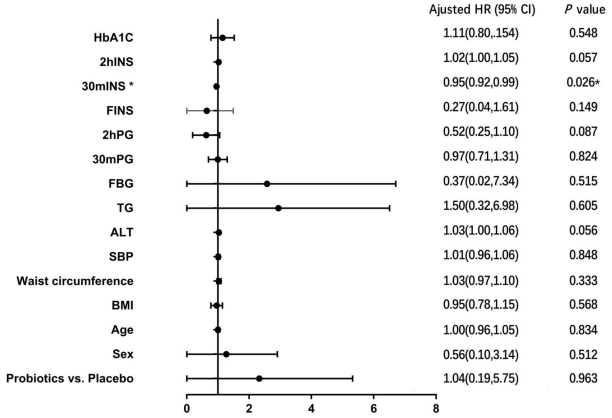


FIGURE 3
COX regression analysis of risk factors for T2DM after 6 years.

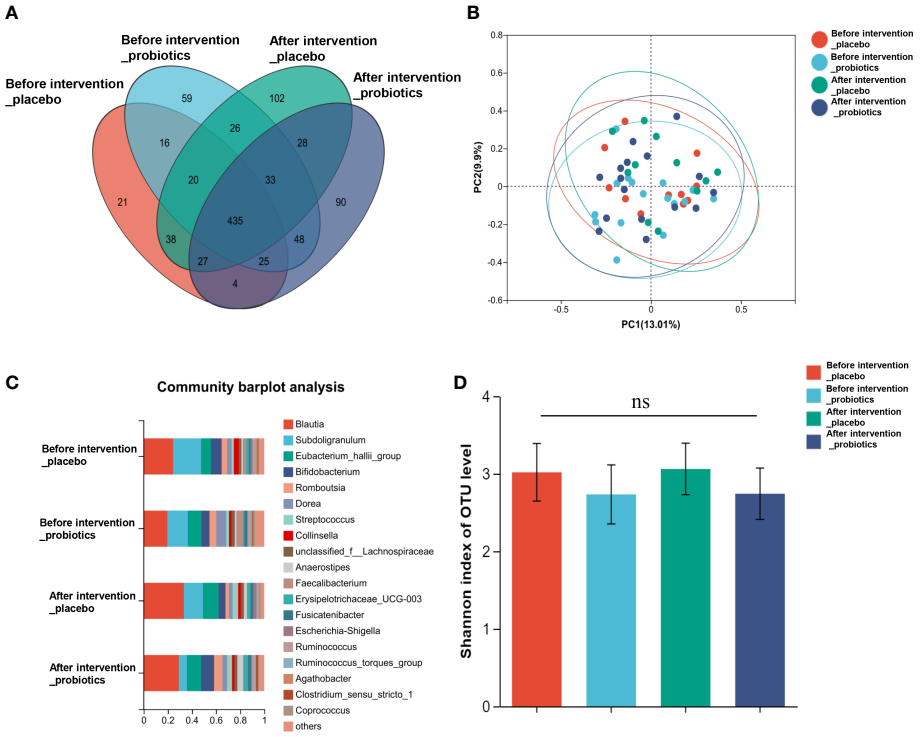


FIGURE 4
Composition and diversity of gut microbiota before and after two years probiotics or placebo intervention. **(A)** The Venn diagram shows the common or endemic species between groups in the level of OUT; **(B)** Weighted UniFrac PCoA; **(C)** Compositional change at the genus level; **(D)** α diversity analysis of gut microbiota.

placebos intervention in any of the groups compared with those before intervention. To assess the differences in bacterial diversity among groups, sequences were aligned for alpha-diversity. No significant difference in the Shannon index between the probiotic group and the placebo group was observed. (Figure 4D). We also compared the difference in gut microbiota's structural composition and abundance changes between the baseline and after intervention among T2DM and non-T2DM groups. The Venn diagram of bacteria showed that 426 of the total 972 genera were shared among the 4 groups (Figure 5A). The results of PCoA showed that the microbiota was similar between the T2DM group and the non-T2DM group both at baseline and after intervention (Figure 5B). Microbial community analysis demonstrated that Blautia, Subdoligranulum, Eubacterium hallii, Bifidobacterium, and Romboutsia accounted for the majority in each group (Figure 5C). At baseline and after the intervention, no significant difference in the Shannon index between the T2DM group and the non-T2DM group was observed. (Figure 5D).

The mean proportion of subdoligranulum and monoglobus in the T2DM group was significantly lower than that of the non-T2DM group both at bas `eline and after the intervention. The proportion of collinsella was lower in the T2DM group (Figure 6A). Further analysis of the specific species microbiota showed that there were no differences among groups in the mean proportion of the metabolic-related microbiota, as well as in produces short-chain fatty acids-related microbiota and gut probiotics (Figures 6B-D) ylogenetic Investigation of Communities using Reconstruction of Unobserved States (Picrust2) software. The results showed that these metabolism-related pathways consisted of carbohydrate metabolism, amino acid metabolism, transcription, replication, recombination and repair, and other metabolic pathways in the non-T2DM and T2DM group (Figure 7).

## 4 Discussion

IGT is closely associated with metabolic disease progression. According to the epidemiological data, about 70% of IGT patients progress to DM within 5 years in China (11). The rapidly growing trend means an urgent need to prevent DM actively. Early dietary modification can prevent the development of diabetes, but it is difficult for individuals to adhere to. The gut microflora plays a crucial role in regulating host metabolism. Changing the composition and/or metabolic activity of gut microflora may contribute to human health. Evidence from human and animal studies suggest that the gut microbiome is a common pathway mediating the therapeutic effects of bariatric surgery, dietary control, and hypoglycemic drug therapy (12–14). Therefore, remodeling the gut microflora may be a new direction for humans to prevent and treat diabetes.

The treatment of metabolic diseases with probiotics is a hot topic in intestinal microbiota research. However, there are fewer studies on probiotics for the prevention and treatment of IGT

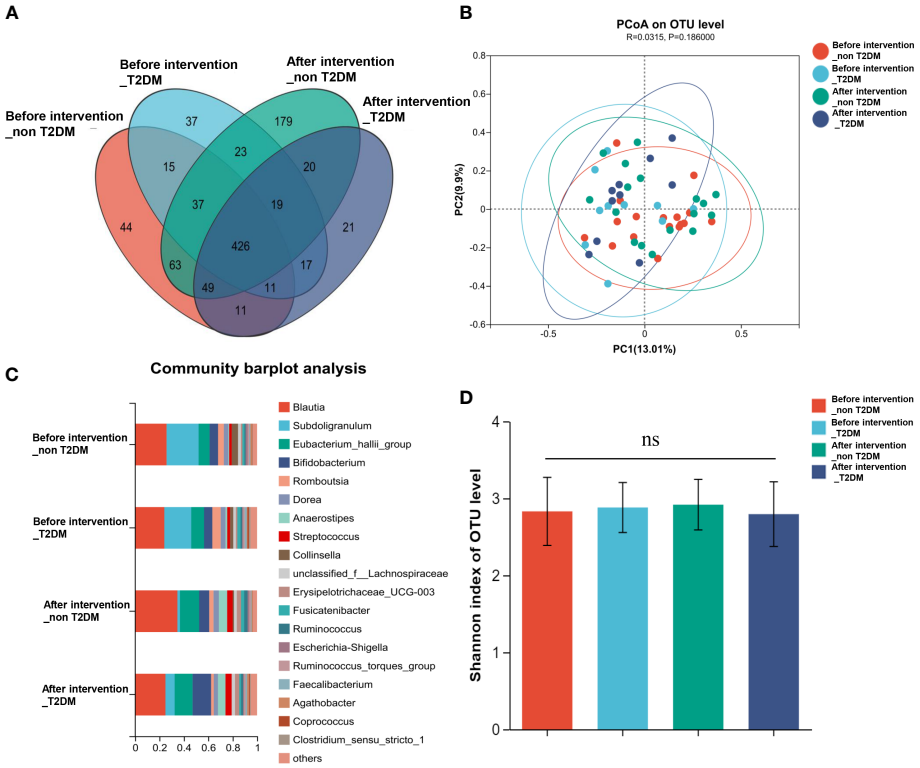


FIGURE 5
Composition and diversity of gut microbiota in T2DM patients and non-T2DM patients before and after two years of probiotics intervention. **(A)** The Venn diagram shows the common or endemic species between groups in the level of OUT; **(B)** Weighted UniFrac PCoA; **(C)** Compositional change at the genus level; **(D)** α diversity analysis of gut microbiota.

patients. We previously observed that probiotics supplementation for IGT patients for 2 years did not significantly reduce the risk of IGT conversion to T2DM in the PPDP study (9). In the present study we intend to observe the impact of early and long-term probiotics supplementation on the conversion of diabetes in a longer time (6 years). This is the first long-term prospective study to analyze the efficacy of probiotic administration on glucose metabolism in IGT subjects. The supplementary probiotics in the PPDP study were provided by Bifico (Approval number: S10950032), an over-the-counter capsule consisting of live combined Bifidobacterium longum, Lactobacillus acidophilus and Enterococcus faecalis. It has been reported that Bifidobacterium longum supplementation can attenuate hyperglycemia, improve the antioxidant capacity of the liver, repair intestinal barrier injury, and reduce inflammation in diabetic mice (15). Lactobacillus acidophilus was also reported that can alleviate T2DM by regulating hepatic glucose, lipid metabolism and gut microbiota in mice (16). In addition, it was indicated that Enterococcus faecalis treatment could improve glucose homoeostasis, increased energy expenditure and reduced hepatic steatosis in the db/db mice fed with high fat (17). However, in the present study, after 6 years' follow up, the Probiotic group showed no significant superiority in preventing the conversion of IGT to T2DM as compared with the Placebo group. Similarly, no significant differences in the diversity and composition of the gut microbiota were observed between the two groups, nor were differences in microbiota observed between groups with or without T2DM. COX regression also showed that probiotics intervention was not affecting IGT conversion to T2DM. Only 30-minute-insulin after glucose loading was the factor affecting the conversion of IGT into T2DM, which indicated that the decrease in islet β -cell function was an important cause of T2DM.

Although the relationship between the gut microbial ecological imbalance and the development of obesity and diabetes is being extensively explored, the conclusions of various studies are different. The results of randomized controlled studies on pregnant women with gestational diabetes or obesity showed that probiotic intervention had no effect on glycemic control, but might improve lipid metabolism (18, 19).In another study of prediabetes adolescents, it was not observed that oral probiotics could improve FBG and HbA1c after 4 months (20). Similarly, in a 24-week probiotic intervention study on adults with prediabetes, the goodness of glycosylated hemoglobin was not observed (21). Our studies are consistent with the conclusion of these studies that probiotics have a limited therapeutic effect on metabolic diseases. However, some studies confirm the beneficial role of gut microbiota in glycemic control and T2DM. Tonucci et al. found that Probiotic consumption improved glycemic control in T2DM subjects (22). The application of a novel probiotic formulation to T2DM showed that the intervention was safe and well tolerated (23). Different probiotic strains, their combinations or the time and duration of intervention may play different roles in the efficacy of the probiotic

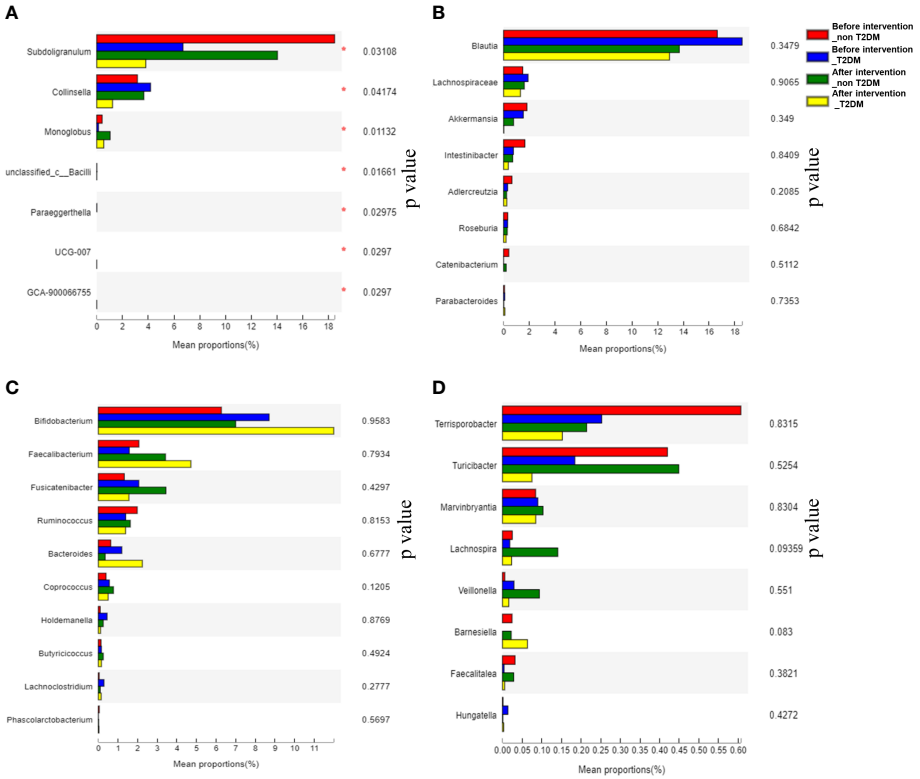


FIGURE 6
Analysis of significantly altered gut microbiota and specific species microbiot in T2DM patients and non-T2DM patients after two years of probiotics intervention. **(A)** Analysis of significantly altered gut microbiota; **(B)** Analysis of metabolism-related microbiota; **(C)** Analysis of producing short-chain fatty acids-related microbiota; **(D)** Analysis of gut probiotics.

intervention on glucose control. The limited sample size and subject-to-subject variability suggest that future studies are needed to confirm and extend these observations.

The gut microbiota profile may be related to and responsive to a particular dietary pattern (24). Therefore, supplementation with beneficial microorganisms such as probiotics and their metabolites may alter microbiota distribution and thus affect metabolic parameters (25). However, in this study, gut microbiota analysis results showed no difference in the composition and diversity of the gut microbiota between the T2DM group and the IGT group after two years of probiotic intervention. This may be related to the small fecal sample size selected in this study and the large individual differences of samples within the same group, or it may be the result of functional variation of the strain, indicating that a more precise strategy is required for probiotic therapy. The analysis of specific microbiota showed that compared with the IGT group, the proportion of subdoligranulum, collinsella and monoglobus in the T2DM group decreased after two years of intervention. The occurrence of T2DM may be related to the changes in the composition of intestinal microbiota. Although there is a lack of

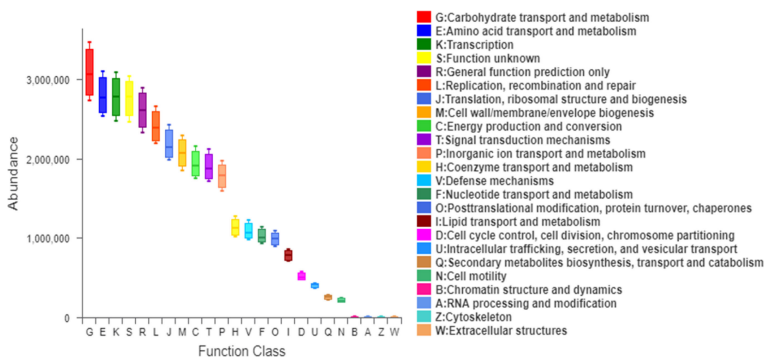


FIGURE 7
Functional prediction and comparison of gut microbiota between T2DM and non-T2DM groups.

consensus on which microbiota are significantly changed in T2DM, a common observation has been a decreased abundance of butyrate-producing bacteria with this condition (26). Subdoligranulum and collinsella have been proven to produce butyric acid (27, 28), and a study has shown that the decrease of Monoglobus may be related to insulin resistance and systemic inflammation (29).

There are some limitations in our study. First, this was a small sample size study that enrolled a limited number of patients with IGT. More clinical and laboratory studies using large-size samples and long-term observation are needed to confirm the role of probiotics in developing IGT into DM. Second, the results of the study of Bifico used in this study as a probiotic supplement for Chinese patients are not representative of the effects of other strains on other people or races. Third, the study did not document lifestyle factors, such as diet and exercise, which might have influenced blood sugar outcomes. There is also no recorded family history of T2DM, which is a very strong risk factor for developing T2DM. However, the placebo control designed in this study could compensate for this effect to the greatest extent. To provide preliminary data that could drive more conclusive testing. Therefore, high-quality, large-scale, multicenter randomized controlled trials with longer follow-up are needed to compare safety and efficacy further.

## 5 Conclusions

Nevertheless, the results of this study suggest that supplementation with active probiotics of Bifidobacterium, Lactobacillus acidophilus and Enterococcus faecalis is safe, although it does not reduce the risk of IGT conversion to DM. More clinical and laboratory studies using large samples and long-term observation are needed to explore the effects of different probiotic strains on IGT. This pilot study was designed to provide preliminary data to conduct more conclusive hypothesis testing.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject [https://www.ncbi.nlm.nih.gov/bioproject/], PRJNA923108.

## Ethics statement

The studies involving human participants were reviewed and approved by the institutional review board of Shanghai East Hospital and was conducted in accordance with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

BF designed the study and oversaw the project implementation. QY conceived and carried out experiments. WH and YT participated in data analyses, interpretation and writing publications. XUL, YY and XIL participated in data collection, data analyses and interpretation and writing publications. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1113611/full#supplementary-material

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# Pharmacomicrobiomics and type 2 diabetes mellitus: A novel perspective towards possible treatment

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Type 2 diabetes mellitus (T2DM), a major driver of mortality worldwide, is more likely to develop other cardiometabolic risk factors, ultimately leading to diabetes-related mortality. Although a set of measures including lifestyle intervention and antidiabetic drugs have been proposed to manage T2DM, problems associated with potential side-effects and drug resistance are still unresolved. Pharmacomicrobiomics is an emerging field that investigates the interactions between the gut microbiome and drug response variability or drug toxicity. In recent years, increasing evidence supports that the gut microbiome, as the second genome, can serve as an attractive target for improving drug efficacy and safety by manipulating its composition. In this review, we outline the different composition of gut microbiome in T2DM and highlight how these microbiomes actually play a vital role in its development. Furthermore, we also investigate current state-of-the-art knowledge on pharmacomicrobiomics and microbiome's role in modulating the response to antidiabetic drugs, as well as provide innovative potential personalized treatments, including approaches for predicting response to treatment and for modulating the microbiome to improve drug efficacy or reduce drug toxicity.

KEYWORDS

**type 2 diabetes mellitus, pharmacomicrobiomics, gut microbiome, antidiabetic drugs, treatments**

## 1 Introduction

Type 2 diabetes mellitus (T2DM), a major cause of morbidity globally, is a complex disease with environmental and genetic risk factors that ultimately can lead to serious complications (1). It is characterized by peripheral insulin resistance (IR) and impaired insulin secretion (2), and is projected to affect up to 783 million people by 2045 (3). Individuals with T2DM have an increased risk of developing diabetic complications

including microvascular events, kidney failure, stroke and limb amputations (4). Although there are several non-pharmacological and pharmacological treatments available for managing T2DM (5, 6), problems associated with potential side-effects and drug resistance remain unresolved.

Over recent years, the human gut microbiota harboring trillions of microbes and other microorganisms forms a complex ecosystem and plays a vital role in health and disease. For instance, gut microbiota functioned as an important contributor in the pathogenesis of obesity and obesity-related metabolic dysfunctions (7). The balance of pathogenic and beneficial bacteria was also reported to be associated with diabetes and cardiovascular diseases (8, 9). Various studies showed the effect of drug intake and drug-induced metabolites on the gut microbiota (10–12), and the gut microbiota could also contribute to an individual's response to several drugs in turn (13, 14).

Pharmacomicrobiomics, a new branch, has been proposed to describe the influence of microbiome variations on drug response (15). It was useful for investigating how the effect of drugs could be modulated by the gut microbiota. In addition, pharmacomicrobiomics played a crucial role in the development of personalized medicine in order to improve the drug efficacy and reduce adverse drug reactions (16). Undoubtedly, the microbiota modulation associated with pharmacomicrobiomics has the potential to enable the development of microbiota-targeting approaches.

In the present review, we summarize microbiome variations in T2DM and highlight how these microbiomes actually play a preponderant role in its development. Besides, we also investigate pharmacomicrobiomics and microbiome's role in modulating the response to antidiabetic drugs, focusing particular attention on innovative potential personalized treatments for T2DM.

## 2 The role of gut microbiota in T2DM

Gut microbiota, known as the "human second genome", consists of the 10–100 trillion microorganisms including bacteria, archaea and viruses (17), and has 150 times larger gene sets than humans (18). It was a well-known fact that the gut microbiota played an crucial role in the proper functioning of human organisms (19). Due to the advancements in sequencing technologies, researches on gut microbiome have developed rapidly during the past decade. Accumulating evidence indicated that gut microbiota dysbiosis contributed to the onset and development of T2DM (20–22).

Although the complete bacterial counts were similar between healthy controls and T2DM patients (23), the diversity was significantly declined in T2DM (10, 24–27). Furthermore, the Integrative Human Microbiome Project found that prediabetic individuals had distinguishable microbial patterns at baseline from the healthy controls (28). Both humans and animal models with T2DM showed the compositional changes in microbiota profiles, especially at the phyla and genus levels (29, 30). A previous study showed a decrease in the abundance of butyrate-producing bacteria and an increase in several opportunistic pathogens, including *Clostridium symbiosum*, *Clostridium hathewayi* and *Escherichia coli* in Chinese T2DM patients (29). Likewise, Li et al. revealed a notable decrease of butyrate-producing bacteria such as *Bifidobacterium* and *Akkermansia*, as well as a significant increase of *Dorea* in Chinese T2DM individuals (31). Another study in Europe found an increase abundance of four *Lactobacillus* species and a reduction in the abundance of five *Clostridium* species in T2DM patients (23). Analogously, a recent study demonstrated that *Lactobacillus* was significantly higher, whereas *Clostridium coccoides* and *Clostridium leptum* were significantly lower in newly diagnosed T2DM patients (32). Furthermore, patients with refractory T2DM revealed reductions in *Akkermansia muciniphila* and *Fusobacterium*, as well as a corresponding enrichment of *Bacteroides vulgatus* and *Veillonella denticariosi* (33). Yassour and his colleagues suggested that decreased *Akkermansia muciniphila* could be used as a biomarker for the early diagnosis of T2DM (34). Notably, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were reported as the main predominant phyla in T2DM patients (27, 35–37). In newly diagnosed T2DM, the phylum *Firmicutes* significantly increased, along with the phylum *Bacteroidetes* significantly decreased (27, 35, 37). Sedighi et al. performed a case-control study and found that *Firmicutes* increased but *Bacteroidetes* decreased in T2DM patients (24). Uniformly, a recent study recruited 65 T2DM patients and 35 healthy controls and observed a consistent result (36). Interestingly, these studies also highlighted a significant increase (36, 37) or decrease (27, 35) in *Proteobacteria* respectively. Therefore, it is necessary to reduce the impact of confounding factors (i.e. dietary habits, lifestyle, disease status) and increase the sample size to further verify these inconsistent results.

In addition, the role of gut microbiota in T2DM was also confirmed in several animal models (38–40). 16S rRNA gene sequencing illuminated that the abundance of several butyrate-producing bacterial genera, such as *Dialister*, *Anaerotruncus* and some members of *Ruminococcaceae*, was reduced in diabetic cats (38). Okazaki et al. established a T2DM zebrafish model and revealed a lower bacterial diversity than the control (39), which indicated functional similarities in T2DM individuals. Wang et al. constructed Zucker diabetic fatty (ZDF) rats that were fed with Purina Lab Diet to induce obesity-related T2DM and found twelve potential biomarkers of microbial flora and 357 differential metabolites in ZDF rats, among which three flora, *Phocea*, *Pseudoflavonifractor* and *Lactobacillus*, contributed to the perturbation of metabolites (40). Besides, microbiome analysis demonstrated that the time-dependent alterations in the fecal microbiome were associated with age and disease progression of T2DM in ZDF rats (41). Of interest, *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, and *Allobaculum* were the most abundant genera in 15-week-old rats (41). Leptin receptor-deficient db/db mice were commonly used as T2DM murine models (42). Yu et al. found a significant increase in *Verrucomicrobia* and a significant decrease in *Bacteroidaceae* in T2DM murine model (43). They also showed that the fecal microbiota transplantation (FMT) from T2DM murine transplanted into pseudo-germ-free mice induced an increase in body weight and fasting blood glucose. Another study exhibited a loss of diurnal oscillations in several certain bacteria, including *Akkermansia*, *Bifidobacterium*, *Allobaculum*, and *Oscillospira* in T2DM db/db mice (44). In high-fat diet (HFD)/streptozotocin

(STZ)-induced T2DM mice model, genistein could alleviate inflammation and IR by increasing the abundance of *Bacteroides* and *Prevotella* and decreasing the levels of *Helicobacter* and *Ruminococcus*, indicating that the gut microbiota might be a potential target for the treatment of T2DM (45). Recently, increasing evidence showed that several bacterial taxa, including *Akkermansia muciniphila* (46) and *Bacteroides* (47), had consistent trends in T2DM patients and animal models. Collectively, gut microbiota is closely related to the onset and development of T2DM (Table 1), as well as may be an important participant in the pathogenesis of T2DM.

As mentioned above, gut microbiota plays a regulatory role in the development of T2DM. There is growing evidence that microbiota and its metabolites are involved in modulating gut permeability, as well as influence immune and inflammatory responses and metabolic homeostasis in T2DM (Figure 1). Intestinal barrier protects the body from intestinal lipopolysaccharide (LPS), and increased intestinal permeability leads to chronic inflammation and is a characteristic of human T2DM (48). A previous study, in turn, verified that hyperglycemia drived intestinal barrier permeability through altering the integrity of tight and adherence junctions (49). Microbial anti-inflammatory molecule derived from *Faecalibacterium prausnitzii* could restore the intestinal barrier structure and function *via* stabilizing the cell permeability and increasing zonula occludens-1 expression in T2DM mouse model (50). *Akkermansia muciniphila*-derived extracellular vesicles (AmEVs) were reported to decrease in the fecal samples of patients with T2DM, and AmEV administration reduced intestinal permeability by enhancing tight junction function and thus improved glucose homeostasis in HFD-induced diabetic mice (51). Strikingly, numerous clinical and preclinical researches have shown that gut microbial imbalance is closely interconnected to IR. For example, an observational study found that reduced fecal *Akkermansia muciniphila* abundance increased the severity of IR in Asians with T2DM, particularly those who were lean in weight (52). Similarly, another study reported that butyrate-producing bacteria, such as *Fecalibacterium prausnitzii*, alleviated IR by inducing glucagon-like peptide-1 receptor (GLP-1) secretion from colonic L cells *via* fatty acid receptor GPR43 (53). Of note, the levels of fecal and serum LPS were elevated in HFD/STZ-induced T2DM model (54). Subsequent studies have confirmed that when LPS is transported to metabolic tissues, it induces a pro-inflammatory response through the activation of toll-like receptor 4 (TLR4) pathway, ultimately leading to IR (55). Moreover, Amuc_1100, a purified membrane protein from *Akkermansia muciniphila*, improved the integrity of the intestinal barrier by interacting with toll-like receptor 2 (TLR2), thus alleviating IR in HFD-fed mice (56). It was well known that microbiota and its metabolites stimulated anti-inflammatory cytokines and decreased inflammatory markers, as well as improved glucose metabolism. For instance, *Lactobacillus plantarum* had potential hypoglycaemic ability and improved glucose metabolism by increasing the levels of interleukin-10 and reducing the levels of malondialdehyde and tumour necrosis factor-α, thus ameliorating IR and systemic inflammation in HFD/STZ-induced T2DM mice (57, 58). Furthermore, *Lactobacillus casei* and *rhamnosus* also decreased the

TABLE 1 The changes of gut microbiota in T2DM.

| Subjects | Methods | Changes in gut microbiota | References |
|---|---|---|---|
| European women with T2DM | Shotgun sequencing | Increased abundance of four *Lactobacillus* species and reduced the abundance of five *Clostridium* species | (23) |
| Iranian T2DM patients | 16S rRNA sequencing | Increased *Firmicutes* and decreased *Bacteroidetes* | (24) |
| Chinese T2DM patients | Deep shotgun sequencing | A decrease in the abundance of butyrate-producing bacteria and an increase in *Clostridium symbiosum*, *Clostridium hathewayi* and *Escherichia coli* | (29) |
| Danish T2DM patients | 16S rRNA pyrosequencing | Reduced the proportions of *Firmicutes* and *Clostridia* | (30) |
| T2DM patients from Northern China | 16S rRNA pyrosequencing | Decreased butyrate-producing bacteria such as *Bifidobacterium* and *Akkermansia*, as well as increased *Dorea* | (31) |
| Newly diagnosed T2DM patients from Taiwan | 16S rRNA sequencing | A higer level of *Lactobacillus* and a lower level of *Clostridium coccoides* and *Clostridium leptum* | (32) |
| Patients with refractory T2DM from Taiwan | 16S rRNA sequencing | Decreased *Akkermansia muciniphila* and *Fusobacterium*, as well as enriched *Bacteroides vulgatus* and *Veillonella denticariosi* | (33) |
| Patients with sub-clinical state of T2DM from Korea | Shotgun metagenomes | Decreased *Akkermansia muciniphila* | (34) |
| Chinese T2DM patients | 16S rRNA sequencing | Increased the abundance of Proteobacteria and the ratio of Firmicutes/Bacteroidetes | (36) |
| Newly diagnosed T2DM patients from India | 16S rRNA sequencing | Decreased *Akkermansia, Blautia*,and *Ruminococcus* and increased *Lactobacillus* | (37) |
| Lean individuals with newly diagnosed T2DM | Shotgun metagenomic sequencing | Decreased the abundance of Akkermansia muciniphila | (46) |

T2DM, type 2 diabetes mellitus; ZDF, zucker diabetic fatty; HFD, high-fat diet.

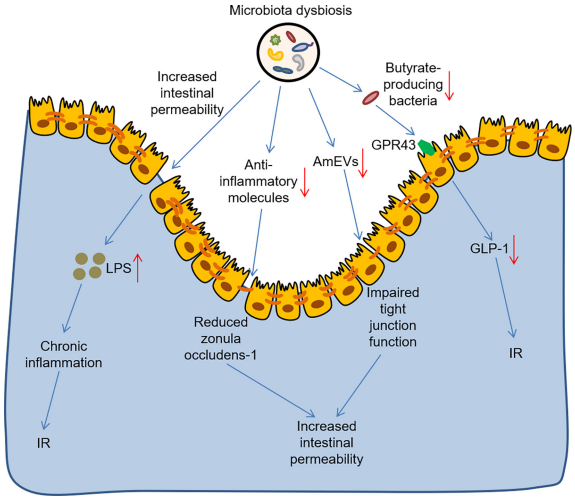


FIGURE 1
The role of gut microbiota dysbiosis in the development of T2DM. Microbiota dysbiosis increased intestinal barrier permeability and increased the level of LPS, thus leading to chronic inflammation and IR. Microbiota dysbiosis reduced the anti-inflammatory molecule and AmEVs levels, thus impairing the intestinal barrier structure and increasing intestinal barrier permeability. Microbiota dysbiosis also reduced butyrate-producing bacteria, which contributed to IR by inhibiting GLP-1 secretion from colonic L cells *via* the fatty acid receptor GPR43. LPS, lipopolysaccharide; IR, insulin resistance; AmEVs, *Akkermansia muciniphila*-derived extracellular vesicles GLP-1, glucagon-like peptide-1 receptor.

levels of the inflammatory markers tumor necrosis factor-α and interleukin-6 in HFD/STZ-induced T2DM rats (59, 60), thereby improving glucose metabolism and attenuating symptoms of T2DM. Although several potential detrimental microbes, such as *Fusobacterium nucleatum* and *Ruminococcus gnavus* could increase several inflammatory cytokines in inflammatory diseases (61, 62), its similar role in T2DM remained to be further investigated. Taken together, more studies are needed to deepen our understanding of the role of gut microbiota in T2DM.

# 3 Pharmacomicrobiomics focuses on T2DM

Given that the preponderant role of gut microbiota in T2DM, there is growing interest in pharmacomicrobiomics and microbiome's role in T2DM. Pharmacomicrobiomic studies have been proposed to describe the bidirectional effects between the gut microbiome and antidiabetic drugs, including metformin, thiazolidinedione (TZD), α-glucosidase inhibitors (α-GIs), sodium-glucose cotransporter 2 (SGLT2) inhibitors, glucagon-like peptide-1 receptor agonists (GLP-1 RAs), dipeptidyl peptidase-4 (DPP-4) inhibitors and traditional Chinese medicines (TCMs), appropriately investigating the interactions between the host, gut microbiome and drug action (Figure 2).

## 3.1 Metformin-microbiome-host interactions

Metformin, the most commonly used glucose-lowering drug, can alleviate patients' hyperglycemia *via* the suppression of hepatic glucose production and the increase of glucose uptake and utilization in adipocytes and muscle cells (63). A vast body of studies revealed that metformin altered the gut microbiota community in T2DM (11, 64–66). A multicenter, randomized clinical trial suggested that metformin ameliorated hyperglycemia and hyperlipidemia in T2DM patients *via* increasing beneficial bacteria, such as *Blautia* and *Faecalibacterium* (64). Another randomized, placebo-controlled study showed that metformin perturbed the gut microbiome in individuals with treatment-naive T2DM (11). The authors also transplanted the fecal samples from donors (treated with metformin for 4 months) to germ-free mice and observed that glucose tolerance was improved by increasing the production of short-chain fatty acids (SCFAs) or altering plasma bile acid composition, suggesting a direct metabolic benefits of metformin. Similarily, Sun et al. demonstrated that *Bacteroides fragilis* was decreased and the bile acid glycoursodeoxycholic acid (GUDCA) was increased in newly diagnosed T2DM individuals treated with metformin, and the benefits of metformin were abrogated in HFD-fed mice colonizaed with *Bacteroides fragilis*, implicating that *Bacteroides fragilis*–GUDCA–intestinal farnesoid X receptor (FXR) axis mediated the glucose-lowering effect of metformin (67). A recent systematic review disclosed that pre-diabetes and newly diagnosed T2DM patients treated with metformin were correlated with increases in specific taxa associated with metabolic control, such as *Enterobacteriales* and *Akkermansia muciniphila* (68). In line with clinical research, studies in animal models further confirmed that metformin increased SCFAs production, reduced circulation lipopolysaccharides and inhibited intestinal proinflammatory signaling activities (65, 69, 70), thus contributing to improving metabolic disoders. Notably, gut microbiota could also mediate the side effects of metformin. Forslund et al. emphasized that a relative increase in abundance of

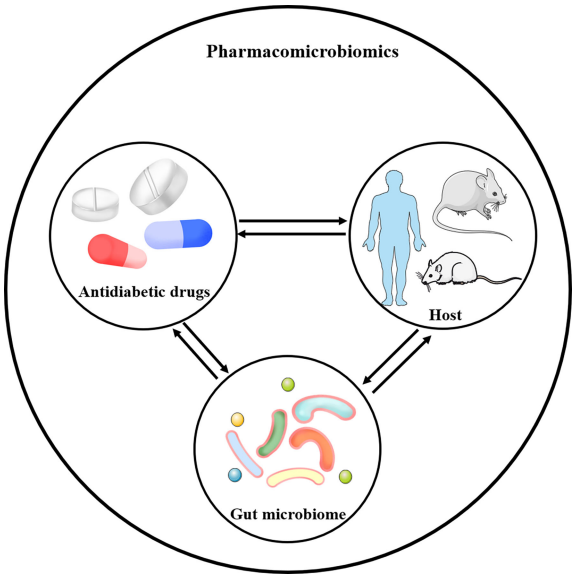


FIGURE 2
Pharmacomicrobiomics studies drug-microbe-host interactions. Antidiabetic drugs-microbe interactions could result in alterations in microbial composition and changes in the chemical structure of compounds, which could in turn directly or indirectly affect the drug response in host, including T2DM patients, mice and rats.

*Escherichia* could enrich virulence factors and gas metabolism genes (10), which contributed to the gastrointestinal side effects of metformin.

## 3.2 TZD-microbiome-host interactions

TZD drugs belong to peroxisome proliferative activated receptor (PPARG) agonists and improve insulin sensitivity for T2DM (71). It reduced hepatic glucose production and increased peripheraltion of glucose and lipid metabolism, thus improving glycemic control. Few studies have discussed the interaction between gut microbiome homeostasis and insulin sensitizers and insulin in T2DM (72, 73). Full-length bacterial 16S rRNA sequencing and RNA sequencing analysis presented that rosiglitazone improved insulin sensitivity without altering the composition of gut microbiome but modifying gene expression signatures associated with lipid and carbohydrate metabolism as well as immune regulation in diabetic mice (72). Moreover, insulin improved taurine and hypotaurine metabolism *via* increasing *Fusobacterium* and up-regulating the genes involved in triglyceride and arachidonic acid metabolism (73).

## 3.3 α-GIs-microbiome-host interactions

α-GIs, including acarbose, voglibose and miglitol, are considered to postpone the digestion of carbohydrates in the intestinal tract and reduce postprandial hyperglycemia in noninsulin-dependent T2DM (74). They are commonly used oral glucose-lowering drugs in China and many Asian countries. A randomized clinical study revealed that acarbose increased the abundance of *Bifidobacterium*, *Eubacterium* and *Lactobacillus*, and lowered the abundance of *Bacteroides* in Japanese patients with T2DM (75). Likewise, in Chinese patients with T2DM, Su et al. found that acarbose treatment increased the content of *Bifidobacterium* and *Enterococcus*, as well as decreased some inflammatory cytokines (76). Mechanistically, Gu et al. highlighted that acarbose altered the relative abundance of microbial genes involved in bile acid metabolism and improved metabolic parameters (12). Interestingly, acarbose also increased the relative abundance of *Ruminococcus* and *Bifidobacterium* in ZDF rats (77). On the other hand, acarbose was an inhibitor of both human and bacterial α-glucosidases, which might limite the ability of the target microbiome to metabolize complex carbohydrates, thus leading to the resistance of acarbose (78). Additionally, due to the weakened microbial enzyme activities, the metabolism of voglibose was reduced, along with significantly glucose-lowering effects were presented in antibiotic pretreatment mice (79), suggesting that gut microbiota mediated the effect of α-glucosidase inhibitors.

## 3.4 SGLT2 inhibitors-microbiome-host interactions

SGLT2 is expressed in the renal proximal tubule and accounts for reabsorbing the filtered glucose. SGLT2 inhibitors exert the glucose-lowering effect by blocking glucose reabsorption in the renal proximal tubule and increasing urinary glucose excretion, accompanied with pleiotropic benefits in cardiovascular and renal protection (80, 81). Several studies have explored the alteration of

gut microbiota with SGLT2 inhibitor treatment (82–85). After a 3-month intervention, empagliflozin improved cardiovascular disease (CVD) risk factors in patients with T2DM, which might be attributed to the significantly altered gut microbiota, including the elevated levels of SCFA-producing bacteria (*Roseburia*, *Eubacterium*, and *Faecalibacterium*) and a reduction in several harmful bacteria (*Escherichia–Shigella*, *Bilophila*, and *Hungatella*) (82). Whereas, van Bommel et al. reported that 2-week treatment with dapagliflozin and gliclazide did not affect either gut microbiome alpha diversity or composition in T2DM patients treated with metformin (83). This discrepancy might be due to the fact that all the participants had already been treated with metformin, which could overshadow the possible impact of dapagliflozin on the gut microbiome. In T2DM mice, dapagliflozin treatment showed a trend for increased *Akkermansia muciniphila* and decreased *Oscillospira* and *Firmicutes*/*Bacteroidetes* ratios (84). However, another study demonstrated that dapagliflozin did not increase the abundance of beneficial bacteria (85). Therefore, more rigorous clincial studies with greater sample size are needed to figure out the interactions between SGLT2 inhibitors and gut microbiota.

## 3.5 GLP-1 RAs-microbiome-host interactions

GLP-1 secreted by enteroendocrine L cells is an incretin hormone and stimulates glucose-dependent insulin secretion. GLP-1 RAs, a new type of hypoglycemic drugs, mimic the effects of endogenous GLP-1, as well as improve glycemic control and cardiovascular outcomes for T2DM patients (86, 87). Accumulating evidence reported that GLP-1 RAs were linked to the changed composition of gut microbiota (88–91). In liraglutide-treated diabetic male rats, several SCFA-producing bacteria, such as *Bacteroides*, *Lachnospiraceae*, and probiotic bacteria, *Bifidobacterium*, were selectively enhanced, which might alleviate systemic inflammation and improve glucose control (88). Besides, germ-free mice colonized with microbiota from liraglutide-treated diabetic mice were shown to improve glucose-induced insulin secretion and regulate the intestinal immune system (91). Also, in T2DM patients, microbial interaction network was altered in patients treated with liraglutide. The distribution of community structure differed between the pre-liraglutide-treatment group (21 species of bacteria were abundant) and post-liraglutide-treatment group (15 species were abundant) (89). Nevertheless, a recent study enrolling 51 T2DM adults with initial therapy of metformin and/or sulphonylureas showed that the diversity and composition of the intestinal microbiota did not change after 12-week liraglutide intervention (92). This inconsistency might be attributed to the initial therapy of metformin and/or sulphonylureas, which could counteract the effect of liraglutide. Recently, Tsai et al. found that gut microbiota contributed to the heterogenicity of GLP-1 RA responses in T2DM patients (90). To sum up, the positive microbial signatures, mainly including *Bacteroides* and *Roseburia*, with immunomodulation effects were dominant in GLP-1 RA responders, while the negative microbial signatures, such as *Prevotella*, *Butyricimonas*, *Mitsuokella* and *Dialister*, with pro-inflammatory properties were dominant in GLP-1 RA non-responders (90). Thus, gut microbiota may be a potential target to improve the GLP-1 resistance.

## 3.6 DPP-4 inhibitors-microbiome-host interactions

DPP-4 inhibitors improve hyperglycemic conditions by stabilizing GLP-1 and glucose-dependent insulinotropic polypeptides (93). A series of studies considered that DPP-4 inhibitors reshaped the microbial composition and increased fecal SCFAs to improve metabolic homeostasis (94–97). Liao et al. demonstrated that DPP-4 inhibitors promoted a functional shift of the altered microbiome induced by HFD, especially increasing the abundance of *Bacteroidetes*, which contributed to improving glucose homeostasis (94). Another study revealed that DPP-4 inhibitors displayed significantly decreased *Firmicutes*/*Bacteroidetes* ratios, and elevated levels of butyrate-producing *Ruminococcus* and *Dorea* in HFD-induced mice (97). Likewise, vildagliptin treatment also reduced the ratio of *Fimicutes*/*Bacteroidetes*, and increased butyrate-producing bacteria, including *Baceroides* and *Erysipelotrichaeae*, in HFD-induced SD rats (96). Furthermore, vildagliptin significantly reduced DPP-4 activity mainly by decreasing *Oscillibacter* and increasing *Lactobacillus* (95), which provided new therapeutic uses of DPP-4 inhibition to tackle gut microbiome dysfunctions in T2DM.

## 3.7 TCMs-microbiome-host interactions

TCMs, known as botanical medicine or phytomedicine, could significantly improve glucose control by enhancing insulin sensitivity, simulating insulin secretion and protecting β-cells (98). In recent years, increasing evidence confirmed that TCMs improved glucose metabolisms and alleviated T2DM at least partly by modulating gut microbiota (99).

A number of studies in animal models of T2DM have extensively explored the interactions between TCMs and gut microbiota (100–107). Zhou et al. found that ginsenoside Rb1, one of the most valuable herbal medicine, increased the abundance of *Parasutterella*, and decreased *Alistipes*, *Prevotellaceae*, *Odoribacter* and *Anaeroplasma* in T2DM mice model, thus attenuating IR and metabolic disorders (100). In T2DM rats model, Baihu Rensheng decoction (BHRS) increased the relative abundance of *Lactobacillus*, *Blautia*, and *Anaerostipes*, as well as decreased the *Allobaculum*, *Candidatus Saccharimonas*, and *Ruminococcus* (101). Mechanically, BHRS was considered to repair gut barrier and inhibit TLR4/NF-κB-mediated inflammatory response. Similarly, Buyang Huanwu decoction (BYHWD), a widely used TCM formula, decreased the

*Firmicutes*/*Bacteroidetes* ratio and increased the abundance of *Lactobacillus* and *Blautia* (102). Another study suggested that Fufang Fanshiliu decoction enriched the abundance of *Lactobacillus*, *Akkermansia*, and *Proteus*, and reduced the abundance of *Alistipes*, *Desulfovibrio*, and *Helicobacter* in T2DM rats model (104). Moreover, Liu-Wei-Di-Huang Pills improved glucose metabolism by promoting the abundance of *Lactobacillus*, *Allobaculum*, and *Ruminococcus*, and increasing SCFAs levels in T2DM rats model (106), which might be related to the SCFAs-GPR43/41-GLP-1 pathway. Shenqi compound (SQC), a TCM formula, has been widely used for T2DM. It showed that SQC exerted a beneficial role by decreasing the *Firmicutes*/*Bacteroidetes* ratio and modulating metabolites in different pathways (107). Gegen Qinlian Decoction exerted the glucose-lowering effect by significantly modulating the overall gut microbiota structure and enriching butyrate-producing bacteria, including *Faecalibacterium* and *Roseburia*, which subsequently attenuated intestinal inflammation (108). Ge-Gen-Jiao-Tai-Wan formula alleviated symptoms ofT2DM rats by increasing the beneficial phylum *Firmicutes* and bile-acid-related genus *Lactobacillus*, thus promoting the production of primary bile acids, and upregulating the PBA-FXR/TGR5-GLP-1 pathway (109). Intriguingly, a current study suggested that the Scrophulariae Radix and Atractylodes sinensis (XC) pair could assist metformin in improving postprandial hyperglycemia by inhibiting the increase of *Bacteroides* in T2DM rats model (105), which could effectively apply to clinical practice in treating T2DM. In addition to the animal studies, a clinical trial in newly diagnosed T2DM patients also underlined that the hypoglycemic effect of berberine was related to the inhibition of DCA biotransformation by *Ruminococcus bromii* (110). Collectively, these findings address the effect of antidiabetic drugs on gut microbiota (Figure 3) and emphasize the host-microbe-drug interactions, providing promising microbiome-targeting approaches to treat T2DM.

# 4 Innovative therapeutics and translational implications of pharmacomicrobiomic studies in T2DM

With the host-microbe-drug interactions in mind, the innovative therapeutics and translational applicability of pharmacomicrobiomics are highly relevant to our understanding of drug efficacy and adverse reactions in T2DM (Figure 4). Given that the magnitude of response to antidiabetic durgs is known to have a unpredictable and high interindividual variability, personalized treatments based on novel technologies and features of the gut microbiome can help to guide a more rational use of these treatments.

## 4.1 Developing predictive tools *via* machine learning and network analyses

With the completion of the human genome and the human microbiome projects (111), a number of large biobanks including gut microbiome and multiple omics data (information on genetics, transcriptome, proteome and metabolome) had been established, such as UK biobank and TwinsUK cohort (112, 113). These biobanks utilized clinical studies, involving well-characterized human cohorts with extensive clinical and demographic details, exploring the host-microbe-drug interactions. In parallel with the existing data, there was continuous need for digging deeper into the unknown filed of drug-microbiome interactions. The accumulated data in literature calls for the construction of predictive tools or models that consider all such parameters to provide accurate predictions (114, 115). Machine learning methods and network analyses, including decision-tree algorithms and random forest, could then be applied to create a predictive tool for the efficacy and

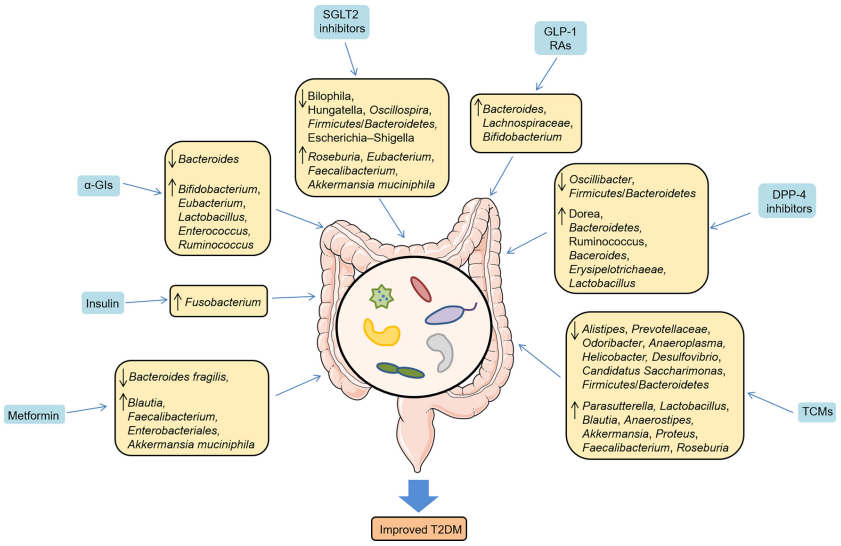


FIGURE 3
Antidiabetic drugs regulated the relative abundance of gut microbes and improved T2DM.

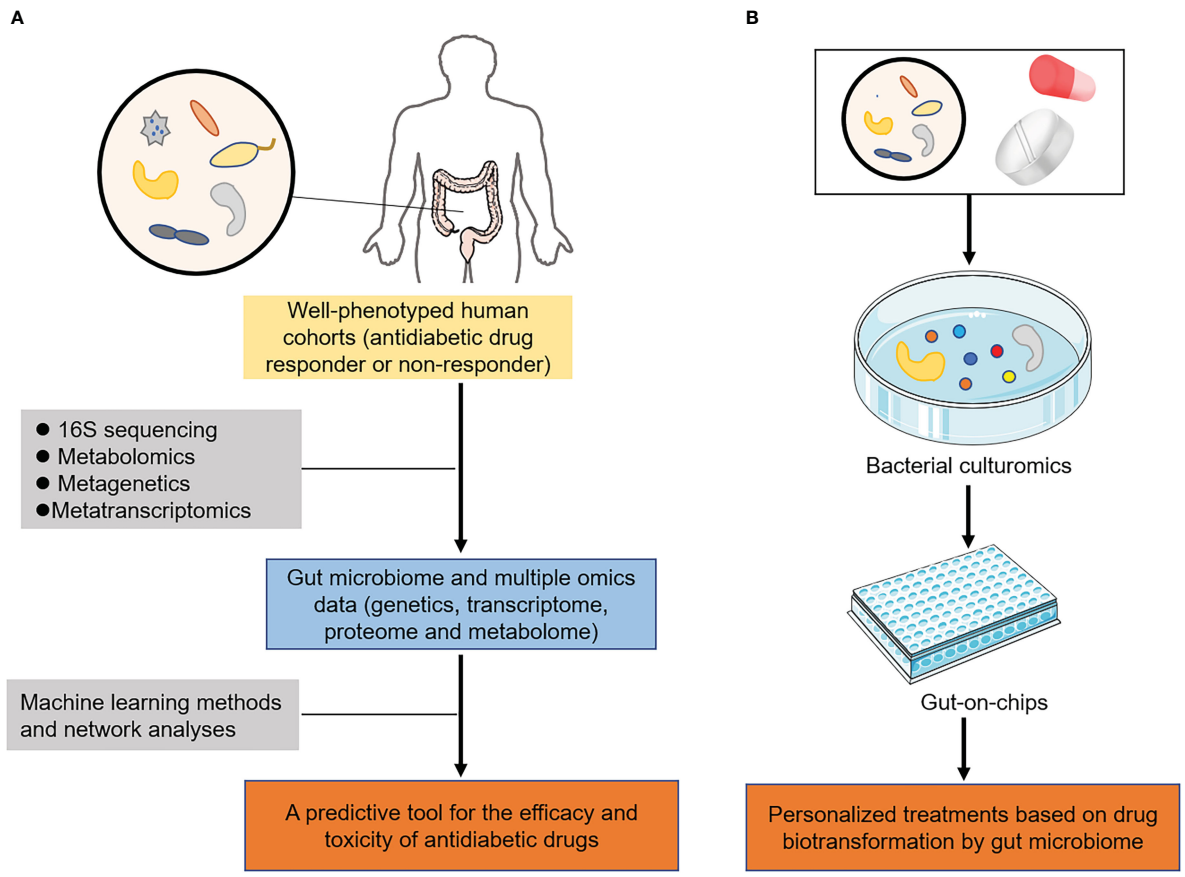


FIGURE 4
Innovative therapeutics and translational implications of pharmacomicrobiomic studies in T2DM. **(A)** In well-phenotyped patient populations, microbial features and multiple omics could be integrated *via* machine learning and network analyses to predict the efficacy and toxicity of antidiabetic drugs. **(B)** Novel technologies, including organs-on-chips and bacterial culturomics assessed their biotransformation by gut microbiome, providing new insights into personalized medicine in T2DM.

toxicity of antidiabetic drugs in T2DM (Figure 4A). For instance, the T2DM prediction model based on the characteristics of the salivary microbiota (microbiome data) was established by random forest in elderly patients with T2DM (116). Another study showed that machine learning tools with gut microbiome profiling exhibited the highest overall predictive power for improving early prediction of T2DM (117). These findings not only had the ability to rapidly inform clinical practice but also elucidated hypotheses regarding the mechanisms in which microbial transformations of drugs changed their pharmacokinetic properties.

## 4.2 Novel technologies for developing personalized treatments

Given the interplay between the host, gut microbiome and drug metabolism, there is increasing awareness that we should take microbiome profile based on novel technologies into account when considering personalized medicine.

Considering that microbiome profiling of human samples provided evidence for microorganism-mediated drug metabolism, further experimental studies are required to identify the specific microbiome responsible for drug biotransformation. Experimental manipulations of gut microbiota incorporated the use of humanized gnotobiotic mice models to further investigate the specific role of the microbiota in modulating drug pharmacokinetics (118). Humanized gnotobiotic mice are typically either germ-free animals or those colonized with defined microbiota and achieved by transplanting human faecal microbiota into germ-free mice (119). As discussed, these models have already proven successful for the treatment of several diseases, including T2DM (120). In recent years, organs-on-chips and bacterial culturomics as emerging technologies also have been developed (121, 122), making functional validation of gut microbiome finally possible. Antidiabetic drugs of interest can be incubated *via* these technologies to assess their biotransformation by gut microbiome, enabling the development of personalized medicine in T2DM (Figure 4B).

# 5 Concluding remarks and future perspectives

There is a mountain of evidence linking gut microbiota to T2DM and its hypoglycemic therapy. In recent years, a growing body of research now focuses on the bidirectional effects between the gut

microbiome and antidiabetic drugs (123, 124). In this review, we summarize the microbe-drug-host interactions and provide a novel perspective towards possible personalized treatment for T2DM.

With the advancement of the studies on the pharmacomicrobiomics (interactions between drugs, microbial communities and host) (125–127), manipulation of microbiota can be a promising target to improve therapeutic outcomes and alleviate adverse drug effects in T2DM. For instance, prebiotics could modulate intestinal microbiota and increase the relative abundance of beneficial bacteria including SCFAs (128, 129), and the combination of hypoglycemic drugs and certain prebiotics could enhance the glucose-lowering effects (130). In addition, FMT, a process of transferring stool from a healthy donor or antidiabetes treatment subjects to mice, displayed a significant improvement in microbial composition and metabolic homeostasis (131). A prospective study revealed that FMT could bring benefits for the management of T2DM *via* modulating levels of certain microbiome such as *Rikenellaceae* and *Anaerotruncus* (132).

Given the great diversity of microbial signatures and the complex drug-microbe-host interactions, a systems-based approach including the integration of multi-omics data with microbiome data and the utilization of bacterial culturomics are required to understand the underlying mechanisms, thus exploring the new therapeutic interventions and potential personalized strategies.

## Author contributions

LJ and SH contributed towards the concept and manuscript writing. BS, YS and CZ revised and supervised overall project. All authors read and approved the final version of manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Targeting the gut microbiota and its metabolites for type 2 diabetes mellitus

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Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia and insulin resistance. The incidence of T2DM is increasing globally, and a growing body of evidence suggests that gut microbiota dysbiosis may contribute to the development of this disease. Gut microbiota-derived metabolites, including bile acids, lipopolysaccharide, trimethylamine-N-oxide, tryptophan and indole derivatives, and short-chain fatty acids, have been shown to be involved in the pathogenesis of T2DM, playing a key role in the host-microbe crosstalk. This review aims to summarize the molecular links between gut microbiota-derived metabolites and the pathogenesis of T2DM. Additionally, we review the potential therapy and treatments for T2DM using probiotics, prebiotics, fecal microbiota transplantation and other methods to modulate gut microbiota and its metabolites. Clinical trials investigating the role of gut microbiota and its metabolites have been critically discussed. This review highlights that targeting the gut microbiota and its metabolites could be a potential therapeutic strategy for the prevention and treatment of T2DM.

KEYWORDS

**type 2 diabetes mellitus, gut microbiota, gut microbial metabolites, targeted therapy, probiotics**

**Abbreviations:** ACA, acetoacetic acids; ATGL, Adipose triglyceride lipase; BA, bile acid; BSEP, bile salts export pump; CA, cholic acids; CDCA, chenodeoxycholic acids; ChREBP, carbohydrate response element binding proteins; CR, Calorie restriction; CYP7A1, Cholesterol 7-alpha hydroxylase; CYP8B1, sterol 12α-hydroxylase; DCA, deoxycholic acid; DIO2, deiodinase type 2; ERK1/2, extracellular regulated protein kinases; FFAR2, Free Fatty Acid Receptor 2; FFAR3, Free Fatty Acid Receptor 3; FXR, farnesoid X receptor; GABAs, γ-aminobutyric acids; GLP-1, glucagon-like peptide-1; GLUT4, glucose transporter 4; GPR109A, G-protein-coupled receptor 109A; HFD, high-fat diet; ICPs, immune checkpoints; IFG, impaired fasting glucose; LCA, lithic bile acid; LPS, lipopolysaccharides; NOD, new-onset diabetes; NTCP, sodium taurocholate cotransporting polypeptide; OATP, Organic Anion Transporting Polypeptide; PAI, propionic acid imidazole; PYY, peotide YY; SCFA, short-chain fatty acid; SHP, small heterodimer; TGR5, Takeda G protein-coupled receptor 5; TLR-4, Toll-like receptor 4; TMA, trimethylamine; TMAO, trimethylamine-N-oxide; UCP1/2/3, uncoupling protein1/2/3; VLCK, very low-calorie-ketogenic.

# 1 Introduction

Diabetes mellitus (DM) is considered one of the most serious public healthcare challenges in the world, with more than 536.6 million people aged 20-79 years (prevalence estimated at 10.5%) reported to have diabetes in 2021. This number is projected to rise to 783.2 million (prevalence estimated at 12.2%) by 2045 (1). Type 2 diabetes mellitus (T2DM), accounting for 90% of cases, is the most prevalent type and is characterized by hyperglycemia and insulin resistance (2, 3). The risk factors that contribute to the onset of T2DM are complex and have not been fully elucidated. Obesity, sedentary lifestyle, and genetic susceptibility are recognized as significant risk factors for T2DM progression (4). An increasing number of studies have shown a clear link between the dysregulated gut microbiota and the development of T2DM (5, 6). Understanding these interactions may lead to novel therapeutic implications for T2DM.

The gut microbiota is a complex and dynamic entity composed of trillions of microorganisms that live in close symbiosis with their host, consisting of hundreds of different species of bacteria, primarily distributed among nine phyla (7–9). It is dominated by the phylum *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria*, which account for 90% of the total human microbiota (10, 11). Gut microbiota is strongly influenced by geographic location, age, lifestyle, diet, and even the mode of birth (12–14). Furthermore, variations in gut microbiota can lead to changes in metabolites, such as bile acids (BAs), branched-chain amino acids (BCAA), short-chain fatty acids (SCFAs), lipopolysaccharides (LPS), trimethylamine (TMA), and propionic acid imidazole (PAI) (15). A study has demonstrated that an increase in trimethylamine-N-oxide (TMAO), a conversion product of TMA in the liver, predicts a high mortality risk in patients with T2DM (16). Although peripheral blood BAs levels do not predict the transition from impaired fasting glucose (IFG) to new-onset diabetes (NOD) (17), Tamara et al. reported a non-absorbable polymeric bile acids chelator (SAR442357) that ameliorated hyperglycemia in preclinical animal models of diabetes by reducing intestinal luminal bile acids levels and delaying the development of DM (18). These clinical reports suggest that gut microbiota and its metabolites may be significantly associated with T2DM progression (19, 20).

Current research generally concludes that gut microbial metabolites can influence the development of T2DM by modulating physiological processes such as β-cell dysfunction, chronic low-grade inflammation, oxidative stress, and dysmetabolism of lipids and glucose (21). For example, SCFAs can decrease the expression of pro-inflammatory cytokines by inhibiting NF-κB activation and IκBα degradation, improving glucose control, and mitigating the development of T2DM (22–24). Conversely, elevated TMAO levels impair glucose tolerance by blocking the hepatic insulin signaling pathway, causing systemic inflammation in adipose tissue, and accelerating the development of diabetes. Although these studies indicate that gut microbial metabolites play a role in the development of T2DM, a systematic summary of the molecular mechanisms involved is still lacking. This review aims to summarize the molecular links between microbiota-derived metabolites and the pathogenesis of T2DM, and discuss recent clinical trials and treatments for T2DM. A better understanding of the interactions between gut microbiota and T2DM could provide insights into T2DM prevention and therapy.

# 2 Diabetes and gut microbial metabolites

Gut microbial metabolites are compounds produced by gut microbiota during the digestion of food. These metabolites, including SCFAs, tryptophan metabolites, TMAO, LPS and BAs, have been shown to play a crucial role in the development of T2DM (25). Most of the metabolites can enter the systemic circulation and act as signaling molecules via various receptors, which further regulate multiple metabolic pathways.

## 2.1 Short-chain fatty acids

SCFAs are major products of the anaerobic fermentation of resistant starch and fiber by the gut microbiota (Figure 1). Only a small fraction of SCFAs in the gastrointestinal tract is taken up from the diet. Butyric, acetic, and propionic acids constitute the most prevalent SCFAs in the body (26). SCFAs can be originally produced by thick-walled flora, including *Clostridium perfringens IV* and *XIV a*. These substances then enter the colonic epithelium via H-dependent or sodium-dependent monocarboxylate transport proteins to provide energy for their production (27). The remaining SCFAs that are then released from the intestine into circulation via the liver and portal system and contribute to the development of several diseases such as obesity, insulin resistance, T2DM, etc. (28).

As members of the fatty acid family, SCFAs can serve as substrates for lipid synthesis. It has been shown that SCFAs can activate AMPK, promote the induction of PGC-1α expression and activate peroxisome proliferator-activated receptor (PPAR), thereby regulating the fatty acid oxidation process (29, 30). Additionally, many studies have also pointed out that important lipid metabolic signals such as cAMP (31), adipose triglyceride lipase (ATGL, the main enzyme of lipolysis) (32), and uncoupling protein (UCP) (33) are also regulated by SCFAs. SCFAs have been found to play a role in hyperglycemic syndrome through G protein-coupled receptors (GPRCs) (34). The most crucial SCFAs receptors are the G protein-coupled receptors free fatty acid receptor 2 (FFAR2), free fatty acid receptor 2 (FFAR3), and G-protein-coupled receptor 109A (GPR109A). Extracellular signal-regulated kinase 1 or 2 (ERK1/2), intracellular calcium activation, cyclic adenosine monophosphate (cAMP), and G protein (Gq or Gi/o) are downstream signaling molecules that FFAR influences the absorption of nutrients (35, 36). FFAR2 (GPR43) is mainly expressed in white adipocytes, islet α and β cells, intestinal enteroendocrine cells, and immune cells (37, 38). Butyrate can inhibit histone deacetylase (HDAC) expression by activating FFAR2, thereby having an inhibitory effect on the inflammatory response (39). FFAR3 (GPR41) is expressed in white adipocytes, immune cells, pancreatic islet α and β cells, and intestinal enteroendocrine cells (40, 41). GPR109A is a G protein-

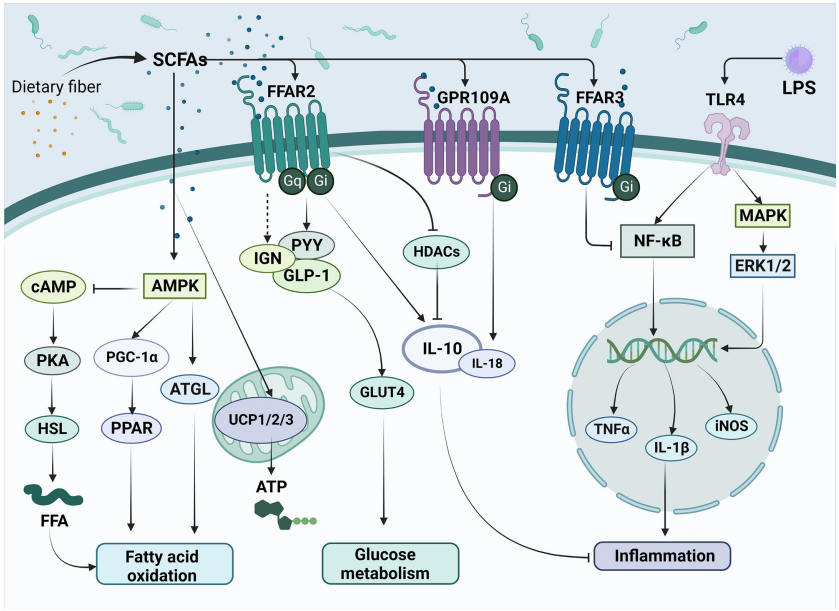


FIGURE 1
The main mechanisms of SCFAs regulating metabolism and inflammation in T2DM. SCFAs are produced by the conversion of dietary fiber by gut microbiota and subsequently enter cells directly or act on transmembrane receptors such as FFAR2, FFAR3 and GPR109A, which are involved in improving T2DM related pathways, such as fatty acid oxidation, glucose metabolism and inflammation response. Meanwhile, SCFAs can inhibit the release of inflammatory factors such as TNF-α and IL-1β triggered by LPS through the NK-κB pathway, thus alleviating the inflammatory response. SCFAs, short-chain fatty acids; FFAR2, Free Fatty Acid Receptor 2; FFAR3, Free Fatty Acid Receptor 3; GPR109A, G-protein-coupled receptor 109A; TLR4, Toll-like receptor 4; LPS, Lipopolysaccharide; AMPK, Adenosine 5'-monophosphate (AMP)-activated protein kinase; cAMP, Cyclic adenosine monophosphate; PKA, protein kinase A system; HSL, hormone-sensitive lipase; FFA, free fatty acid; PGC-1α, Peroxisome proliferator-activated receptor-γ coactivator-1α; PPAR, peroxisome proliferator activated receptor; ATGL, Adipose triglyceride lipase; UCP1/2/3, uncoupling protein1/2/3; ATP, Adenosine triphosphate; IGN, intestinal gluconeogenesis; PYY, peotide YY; GLP-1, glucagon-like peptide-1; GLUT4, glucose transporter 4; HDACs, Histone Deacetylases; IL-10, Interleukin-10; IL-18, Interleukin-18; NF-κB, nuclear factor kappa-B; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular regulated protein kinases; TNFα, Tumor necrosis factor α; IL-1β, Interleukin-1β; iNOS, Inducible nitric oxide synthase.

coupled receptor for nicotinate and has poor sensitivity for butyrate (42, 43).

SCFAs have been extensively studied in the field of metabolic diseases. In a previous study, it was demonstrated that propionate upregulated peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) expression in the colonic tissue, leading to weight loss and significantly reduced blood glucose levels (44). SCFAs activate FFAR2 on enteroendocrine L cells, thereby enhancing the release of GLP-1 and PYY (45). FFAR3 is expressed in vagal sensory neurons and cross-talks with cholecystokinin (CCK) to alter food intake (46). By regulating AMPK, GPR109A promotes Nrf2 nuclear import and induces autophagy, resulting in anti-inflammatory effect (47). It also regulates lipid metabolism and inhibits lipolysis in adipose tissue (48). A recent study also shows that SCFAs may contribute to the development of diabetes through DNA methylation (49).

## 2.2 LPS

Lipopolysaccharide (LPS) is an important feature on the cell wall of gram-negative bacteria and plays an important role in the pathogenesis of T2DM. LPS exhibits an interactive relationship with SCFAs (Figure 1) (50, 51). The amount of LPS can be used to predict the development of many inflammatory diseases associated with participation in natural immunity (52). It has been shown that ecological dysregulation due to high fat intake also upregulates LPS concentrations, resulting in the release of TNF, IL-1, and IL-6 and systemic inflammation (53). The development of endotoxemia will trigger the host's immune response, entering a pro-inflammatory state, which may contribute to metabolic diseases, such as T2DM.

Toll-like receptor 4 (TLR-4) has been identified as an important receptor of LPS, which belongs to a family of transmembrane receptors. Upon TLR-4 activation, transcription of inflammatory cytokines such as TNF-α, IL-1, and IL-6 are enhanced via NF-κB and MAPK pathways. These inflammatory cytokines are significantly elevated in patients with T2DM, subsequently resulting in insulin resistance and pancreatic β-cell dysfunction (54).

## 2.3 Bile acids

Bile acids (BAs), including chenodeoxycholic acids (CDCA) and cholic acids (CA), are synthesized in liver from cholesterol (55). There are two pathways of BAs synthesis: the classical pathway and the alternative pathway. CDCA is effectively catalyzed by mitochondrial sterol 27-hydroxylase (CYP27A1) for oxygenation of the carbon chain of corticosteroids, while the production of CA is determined by sterol 12-hydroxylase (CYP8B1) (56). The bile salts

export pump (BSEP) then secretes bile salts that have been coupled with the amino-acid taurine or glycine into the digestive system, where they are converted into secondary BAs by gut microbiota. In the intestine, CA and CDCA can be converted into deoxycholic acids (DCA) and lithocholic acid (LCA) respectively by the action of bacterial bile salt hydrolase (BSH) and 7α-dehydroxylase enzyme (57). *Clostridium perfringens* is a bacterium that is capable of synthesizing the 7α-dehydroxylase enzyme (58). BSH is an enzyme produced by various strains of gut microbiota, including *Staphylococcus, Neococcus, Enterococcus, Bifidobacterium, Clostridium perfringens, and Parasiticum* (59). BAs are signaling molecules, which regulate insulin sensitivity and inflammation in T2DM via farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) (Figure 2) (60–62). Meanwhile, BAs are also the ligands of vitamin D receptor (VDR) (63), progesterone X receptor (PXR) (64), membrane receptor sphingosine-1 phosphate receptor 2 (S1PR2) (65, 66) and play significant role in regulating inflammation and immune functions.

FXR is mainly expressed in the liver and intestine, and CDCA is the most potentially endogenous agonist of FXR (67). Activation of intestinal FXR induces the expression and secretion of fibroblast growth factor (FGF)15/19, which subsequently enters into liver via enterohepatic circulation (68). Serum FGF15/19 activates hepatic FGF receptor 4 (FGFR4)/-klotho complex, which in turn inhibits cholesterol 7-alpha hydroxylase (CYP7A1) transcription and reduces bile acid synthesis (69, 70). Additionally, it has been reported that clostridia-rich microbiota can promote BAs synthesis by suppressing intestinal FGF19 production (71). Activation of hepatic FXR promotes transcriptional activity of the small heterodimer (SHP), which in turn represses the expression of CYP7A1 expression and reduces bile acid synthesis. One of the downstream targets of FXR is insulin receptor substrate 1 (IRS1)-AKT-phosphatidylinositol 3 kinase (PI3K) pathway, which plays a crucial role in insulin signaling. While both intestinal and hepatic FXR signaling are involved in regulating bile acid homeostasis, they have distinct functions in lipids synthesis and absorption (72). Semi-synthetic bile acid, such as obeticholic acid (OCA), has been shown to be 30 times more effective in activating FXR than CDCA (73). OCA has been found to inhibit bile acid production, improve oxidative stress and liver fibrosis, and decrease hepatic cholesterol and triglyceride content (74, 75). In a study by Sunder et al. (NCT00501592), patients treated with 25 mg OCA showed that insulin sensitivity increased by 28.0% from baseline (76). These studies suggest that OCA may serve as a novel target in alleviating liver inflammation and insulin resistance.

TGR5 (also known as Gpbar-1) is a G protein-coupled receptor. TGR5 is widely expressed in various tissues, including the liver, adipose tissue and intestine, and plays important roles in regulating energy metabolism. TGR5 activation in enteroendocrine L cells can increase glucagon-like peptide-1 (GLP-1) secretion, leading to

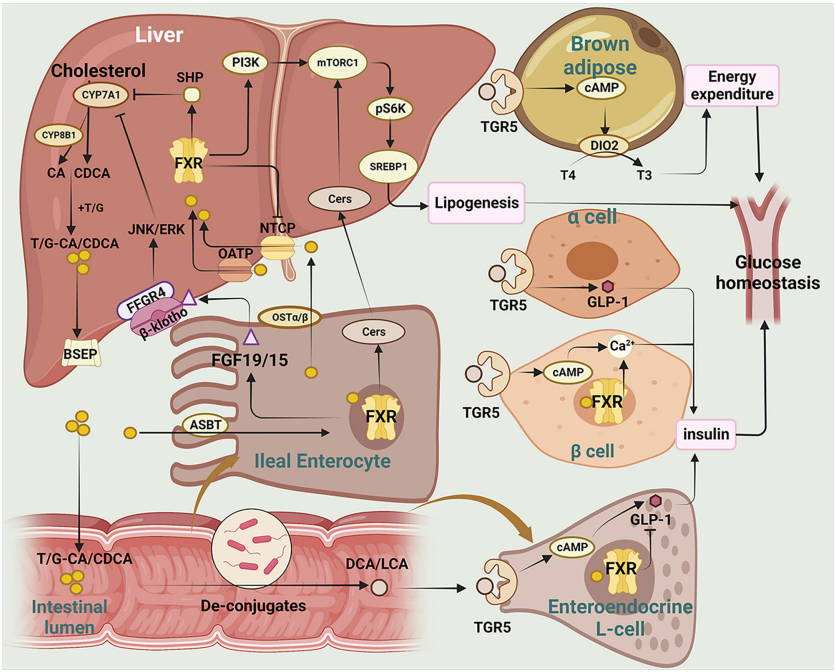


FIGURE 2
The main mechanisms of BAs regulating glucose homeostasis in T2DM. This figure illustrates the metabolism and transformation of bile acids in the liver, intestine, pancreas, and brown adipose tissue, and the mechanisms by which they regulate glucose homeostasis through the two major bile acid receptors, FXR and TGR5.CYP7A1, Cholesterol 7-alpha hydroxylase; CYP8B1, sterol 12α-hydroxylase; CA, cholic acid; CDCA, chenodeoxycholic acid; T/G, taurine/glycine; BSEP, bile salt export pump; SHP, small heterodimer; JNK/ERK, c-Jun N-terminal kinase/extracellular regulated protein kinases; FFGR4, FGF receptor 4; FGF19/15, fibroblast growth factor 19/15; ASBT, apical sodium-dependent bile acid transporter; PI3K, phosphatidylinositol-3-kinases; Akt, protein kinase B; mTOR, mammalian target of rapamycin; Cers,cermides;SREBP1, sterol-regulatory element binding proteins 1; FXR, farnesoid X receptor; NTCP, sodium taurocholate cotransporting polypeptide; OATP, Organic Anion Transporting Polypeptide; OSTα/β, organosolute transport proteins α and β; DCA, deoxycholic acid; LCA, lithic bile acids; TGR5, Takeda G protein-coupled receptor 5; cAMP, Cyclic adenosine monophosphate; DIO2, deiodinase type 2; T4, thyroxine; T3, triiodothyronine; GLP-1, glucagon-like peptide-1.

improved glucose homeostasis and insulin sensitivity (77). TGR5 activation in adipose tissue induces the expression of thyroid hormone deiodinase type 2 (DIO2), which converts inactive thyroxine (T4) into active thyroid hormone (T3) and enhances energy expenditure (78, 79).

Bile acid binding resins and bile acid chelator are used to regulate the BAs pathway (80). Bile acid binding resins work by binding to bile acids in the intestine, preventing their reabsorption and promoting their excretion in the feces. This leads to a reduction in the amount of bile acids in circulation, which in turn stimulates the liver to synthesize more bile acids from cholesterol. A clinical study of 40 Japanese patients with T2DM (NCT038934220) found that colestimide altered bile acid composition and increased the CA ratio, which enhanced energy metabolism, improved blood glucose levels, and alleviated diabetes via TGR5-cAMP-Dio2 pathway (81, 82). Berberine ursodeoxycholate (BUDCA) (83) has been shown to improve glycemic control and lower serum LDL-cholesterol level (NCT03656744) (84, 85). However, it should be noted that bile acid chelator may decrease the hydrophobicity of BAs and increase the risk of gallstone formation (86).

## 2.4 Tryptophan metabolites

Tryptophan is an essential amino acid and can be transformed by gut microbiota into molecules, such as indole and its derivatives, including indole-3- lactate (ILA), indole-3-propionic acid (IPA) and indole-3-acetaldehyde (IAld) (Figure 3). The tryptophan metabolites have been implicated in the pathogenesis of T2DM (87, 88). Indole stimulates GLP-1 secretion from intestinal L cells, resulting in insulin release and reduced blood glucose levels. IPA has been shown to have anti-inflammatory and antiseptic properties by acting on the aryl hydrocarbon receptor (AhR) (89–91). *In vivo*, administration of indole decreased hepatic steatosis and inflammation in rats fed high fat diet. And indole decreased lipid accumulation and stimulates inflammatory responses *in vitro* (92). Activation of AhR by tryptophan metabolites has been shown to have a variety of physiological effects, including regulation of immune responses, inflammation, and cell differentiation (93).

## 2.5 Trimethylamine N-oxide

Trimethylamine N-Oxide (TMAO) has been implicated in the pathogenesis of T2DM and related complications (94). Studies have shown that TMAO may contribute to the development of T2DM by promoting insulin resistance, impairing glucose tolerance, and inducing inflammation (95, 96). High level of TMAO may be associated with mild cognitive impairment, cardiovascular events in patients with T2DM (97–99).

TMAO is produced by gut bacteria from dietary nutrients such as egg and meat products (100). In intestine, gut microbiota breakdown choline, carnitine, or betaine into trimethylamine (TMA) and dimethylamine (DMA), which are absorbed into the bloodstream and transported to the liver (95).. In the liver, TMA and DMA are oxidized by the enzyme flavin-containing monooxygenase 3 (FMO3) to produce TMAO (101). Notably, a diet high in animal-based foods is associated with higher TMAO levels. The increase in circulating TMAO is thought to be possibly related to several factors, including: 1) dietary choline or carnitine content, 2) kidney function, 3) liver function, and 4) gut microbiota composition.

Many studies are focusing on regulating the TMA lytic enzymes to reduce TMAO levels. For example, 3,3-dimethyl-1-butanol

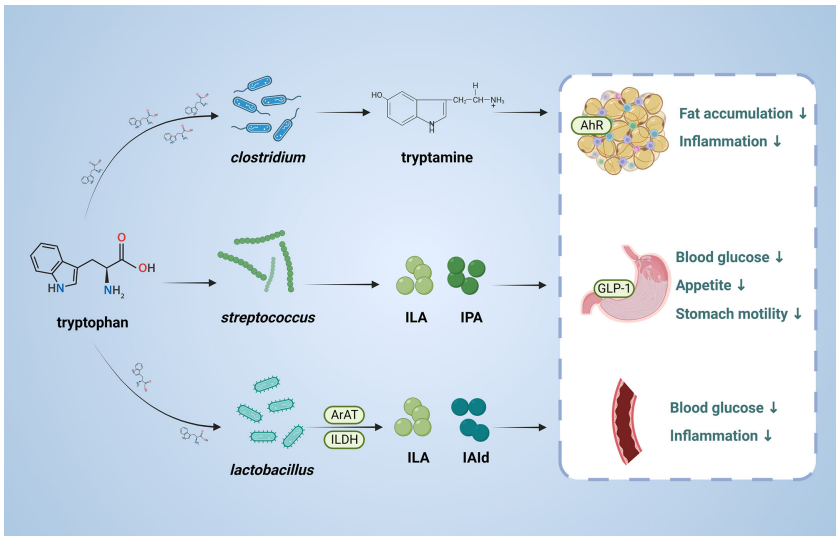


FIGURE 3
Role and mechanism of tryptophan metabolic pathway associated with T2DM. This figure depicts the conversion of tryptophan through the action of the gut microbiota, the products of which are involved in T2DM. ILA, indole-3-lactate; IPA, Indole 3-propionic acid;ILDH, indole-3-lactate dehydrogenase;ArAT Aromatic amino acid aminotransferase, IAld, indole-3-acetaldehyde; AhR, aryl hydrocarbon receptor; GLP-1, glucagon-like peptide-1.

(DMB), which is a structural analog of choline, can inhibit microbial TMA formation. It has been shown that DMB significantly reduces TMAO levels in mice fed a high choline or carnitine diet, thereby inhibiting diet-enhanced atherosclerosis (102). Metformin has also been found to decrease TMAO concentration in db/db mice (103). In addition, it has been demonstrated that berberine reduces TMAO levels by regulating TMA lytic enzymes via remodeling gut microbiota (104).

Antagonist against TMAO, another metabolite of the gut microbiota, has also been shown to improve glucose homeostasis and metabolism disorders (105). Taurisolo, a novel grape pomace polyphenolic extract, significantly decreased serum TMAO levels in healthy subjects (106). It implies that polyphenols can lower TMAO levels, thereby alleviating impaired glucose tolerance and improving adipose tissue inflammation in patients with T2DM (107).

TMAO has been implicated as a novel risk factor for cardiovascular events related to obesity and T2DM. Targeting gut microbiota and TMAO production may serve as potential therapeutic approaches for the treatment of T2DM.

# 3 Regulation of gut microbiota and its metabolites for T2DM therapy

As we gain a deeper understanding of the relationship between gut microbiota and T2DM, more and more therapies are emerging that aim to regulate the gut microbiota and its metabolites (Supplementary Table 1). Recent approach to regulate gut microbiota for T2DM therapy focuses on probiotics, prebiotics, synbiotics, fecal microbial transplantation, diet intervention, bacteriophages, microbiota-targeted drugs and postbiotics (Figure 4).

## 3.1 Probiotics, prebiotics, synbiotics

T2DM has been linked to dysbiosis of gut microbiota (108). Probiotics such as *Bifidobacterium, Lactobacillus*, prebiotics such as oligofructose and inulin, as well as synbiotics (a combination of the two) all play a significant role in the development of T2DM.

Probiotics are live microorganisms that provide beneficial effects to the host when adequately administered. Probiotics have been shown to improve glucose metabolism and insulin sensitivity in patients with T2DM. A combination of *Bifidobacterium lactis LMG P-28149* and *Lactobacillus rhamnosus LMG S-28148* increased PPARγ expression and enhanced insulin sensitivity in high-fat diet (HFD) induced obese mice (109). It has been shown that *Bifidobacterium longum* and *Lactobacillus* upregulated GLP-1 and IL-10 expression in patients with obesity or T2DM, and suppressed lipid accumulation in adipocytes (3, 110). In addition, *Lactobacillus fermentum MCC2760* increased the expression of glucose transporter 4 (GLUT4), GLP-1 and ZO-1, improving glucose tolerance in HFD mice (111).

Inulin is a type of prebiotic fiber that cannot be digested by the human body. It has been demonstrated that inulin is fermented by microbiota to produce SCFAs in the colon (112, 113). In a clinical trial (NCT02009670), consumption of inulin promotes SCFAs production and improves lipid oxidation, resulting in a significant improvement in glycemic control (114). Another study (NCT00750438) shows that inulin-propionate ester supplementation significantly increases colonic propionate levels, and prevents weight gain by promoting GLP-1 secretion (115).

Synbiotics, which combine probiotics and prebiotics, have the potential to provide more significant benefits than when used separately. For instance, when *Lactobacillus* paracasei *N1115* was

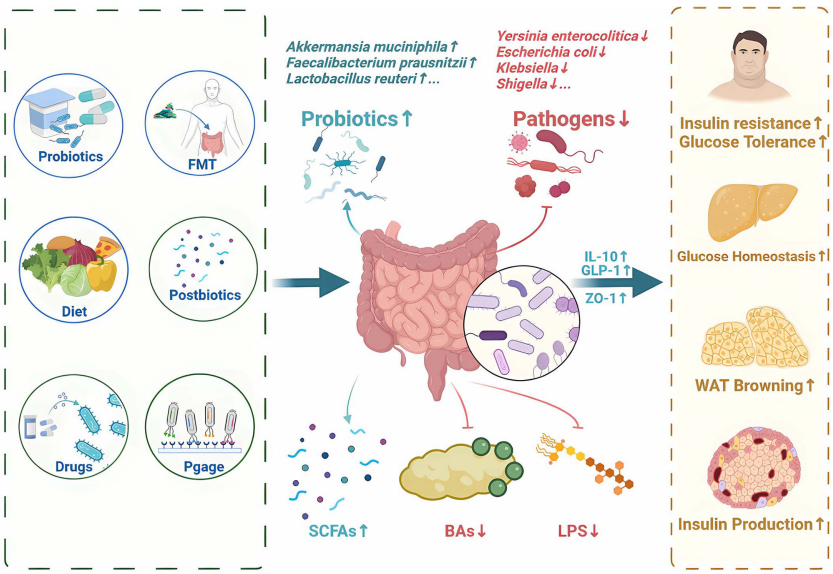


FIGURE 4
Potential therapy and treatments for T2DM by regulating gut microbiota and its metabolites. Recent approaches to regulate gut microbiota for T2DM therapy focuses on probiotics, prebiotics, synbiotics, fecal microbial transplantation, diet intervention, bacteriophages, microbiota-targeted drugs and postbiotics. SCFAs, short-chain fatty acids; FMT, Fecal Microbiota Transplantation; BAs,bile acids; LPS, Lipopolysaccharide; IL-10, Interleukin-10; GLP-1, glucagon-like peptide-1; ZO-1, zonula occludens-1.

combined with oligofructose, it was observed to down-regulate the expression of TLR4 and NF-κB, while up-regulating the p38 MAPK pathway (116). It is important to note that synbiotics currently lack FDA statements, and further clinical validation is required to determine the optimal ratio of probiotics and their safety and efficacy.

## 3.2 Fecal microbiota transplantation

Despite probiotics have been shown to have a potential role for T2DM, fecal microbiota transplantation (FMT) has advantage of entire gut microbiota transplantation. FMT has been recommended for the prevention of chronic *Clostridium difficile* infections since 2013, and it has also shown beneficial effects in ulcerative colitis and even metabolic diseases such as T2DM (117).

Studies have demonstrated that FMT treatment in mice reduces glucose levels, improves insulin sensitivity, and reduces islet cell apoptosis (117). Transplantation of normal human fecal flora into diabetic mice was reported to ameliorate glucose disorders by altering bacterial composition to produce more SCFAs and stimulating GLP-1 releasing via GPR43 receptor (118, 119). In contrast, mice transplanted with gut microbiota from patients with T2DM were found to disrupt blood glucose by regulating BAs metabolism (120). Study by Anne et al. reported that transplanting gut microbiota from lean donors to patients with T2DM could improve insulin sensitivity (121). Similarly, Su et al. showed that the predominant gut microbiota of T2DM patients shifted from *bacteroides* to *Prevotella* after FMT (122), with a significant increase in beneficial organisms (e.g., *bifidobacteria*) and a significant decrease in harmful organisms (e.g., *Bilobacteria*) in a 90-day open-label controlled trial (122). However, it should be noted that FMT may be ineffective or even cause side effects due to the complex composition of the gut microbiota. Elaine et al. reported that FMT had no clinically significant metabolic effects in a clinical study (NCT02530385) (123), possibly due to the small sample size of the trial. Adverse events such as diarrhea, constipation, abdominal pain, and infections have also been reported with FMT (124, 125).

Although FMT is a promising treatment for T2DM, more convincing evidence is needed to confirm the source of donors and frequency of FMT. The adverse effects of dangerous bacteria in the flora, the resilience of the gut microbiota, and the uncertain clinical result of microbiota modifications need more investigation (126, 127).

## 3.3 Diet interventions

A healthy diet helps patients with T2DM improve glycemic control. Research indicates that a weight loss about 15 kg induced by calorie restriction (CR) lead to remissions of T2DM in about 80% patients with obesity and T2DM (128, 129).

In the high-fat diet (HFD) group, an increase in LPS and TMAO and a decrease in SCFAs have been observed, which can affect the host metabolism and immunity. The significant elevation of *Escherichia coli*, *Klebsiella*, and *Shigella* in the HFD group and the decrease of *Lactobacillus* and *Lactobacillus* may provide an early warning for the development of T2DM. However, HFD can lead to an increase in the number of β-cells and induce a decrease of islet infiltration, protecting from the development of diabetes (130). This phenomenon may be related to impairment of immune checkpoints (ICPs) and reduced T-cell attack on pancreatic β-cells, which requires further investigation (131).

Interventions such as calorie restriction (CR), very low-calorie-ketogenic (VLCK), and fasting-mimicking diets (FMDs) have been utilized in metabolic diseases such as obesity and T2DM. CR was found to alter the microbiota and reprogram the metabolism, resulting in a different serum bile acid profile characterized by elevated ratio of non-12α-hydroxylated bile acids (132). The mechanism of CR induced glucose homeostasis may be related to GLP-1 secretion via TGR5/cAMP signaling pathway (133). Additionally, CR can reshape the gut microbiota composition and promote SCFAs production to exert anti-inflammatory effect. VLCK may induce elevating plasma concentrations of acetoacetic acid (ACA) and β-hydroxybutyric acid (β-OHB), and activation of white adipose tissue (WAT) lipolysis (134, 135). Ketogenic diets also alter the gut microbiota and reduce inflammatory Th17 cells (136). These studies indicate that more personalized diet interventions may be utilized for prevention and treatment of T2DM.

## 3.4 Bacteriophages

Gut microbiota contains not only bacteria but also a large number of viruses (dominated by bacteriophages) (137). Bacteriophages specifically infect bacteria in a host-specific manner and are associated with metabolic diseases. For example, altered viral taxonomic composition and reduced viral-bacterial correlation were observed in patients with obesity and T2DM (137, 138). In a previous study, the fecal virome from mice on a low-fat diet was transplanted into the intestine of mice on a high-fat diet. It was observed that the obese mice gained weight more slowly, and their glucose tolerance remained similar to that of mice on a low-fat diet (139). Bacteriophages therapy have been demonstrated to improve clinical healing of diabetic wounds and have less severe impact on the ecosystem than antibiotics (140).

A growing number of studies highlight the possibility that bacteriophages might modify their host genetics through the lysogenic pathway, leading to either an increase or decrease of metabolites levels. For instance, the abundance of *Klebsiella phage* (*vB KpnP SU552A*) was found to be negatively correlated with tryptophan levels, indicating that targeting the tryptophan metabolic pathway by phages could regulate indole derivatives and potentially inhibit AhR to prevent insulin resistance (89–91, 141). However, further research is required to fully understand the role of bacteriophages in the treatment of T2DM, including larger clinical studies to confirm their efficacy.

## 3.5 Microbiota-targeted drugs

Microbiota-targeted drugs are a newly proposed class of drugs that aim to modulate the metabolites of gut microbiota. However, direct targeting of metabolites can have a significant effect on

gastrointestinal function in clinical practice. Therefore, researchers are investigating how to target specific gut microbiota without affecting the gastrointestinal function.

Gut microbiota-derived metabolites play a central role in the host-microbe crosstalk (142, 143). Using a mini-gut model to screen drugs, THIP hydrochloride, methenamine, and mesna have been identified as promising new gut microbiota therapeutics (144). Amuc _1100, a specific outer membrane from *Akkermansia muciniphila*, has been shown to improve metabolism, insulin resistance and dyslipidemia (145, 146). THIP hydrochloride also has the effect of reducing the inflammatory response by decreasing the overgrowth of *Akkermansia muciniphila*, which can cause damage to the intestinal barrier. Urotropine significantly enhances the abundance of *Veillonellaceae*, which converts lactate into SCFAs (147). In addition, Mesna has been shown to decrease the number of *Verrucomicrobiaceae* and *Akkermansia muciniphila* while enhancing SCFA synthesis and decreasing endotoxin production. These changes may contribute to alleviating oxidative stress levels and chronic inflammation (148–150).

### 3.6 Postbiotics

Postbiotics are the byproducts of the metabolic processes of probiotic bacteria, including exopolysaccharides, γ-aminobutyric acid (GABA), and extracellular vesicles (EV) (151). For example, exopolysaccharide has been found to inhibit adipogenesis and pancreatic α-amylase by activating the AMPK signaling pathway (152, 153). It has been reported that GABA improves glucose intolerance, β-cell mass, and inflammatory response (154–156). EV from *Aeromonas aeruginosa* was found to improve intestinal barrier function and glucose tolerance in HFD-induced T2DM mice (157). Meanwhile, in this paper, we use a table (Supplementary Table 2) to summarize in as much detail as possible some information about completed or ongoing gut microbial metabolites clinical trials to show the latest progress of current gut microbial metabolites clinical studies.

## 4 Conclusion

In this review, we discuss the interaction between microbiota-derived metabolites and gut microbiota and their role in T2DM. Currently, there is growing interest in targeting the gut microbiota and its metabolites as a potential therapeutic approach for T2DM. Many approaches have been explored, including the use of probiotics, prebiotics, synbiotics, postbiotics, FMT, dietary interventions, bacteriophages, and microbiota-target drugs.

However, there are still several challenges that need to be addressed. One of the main challenges is the lack of a comprehensive understanding of the complex interactions between the gut microbiota, its metabolites, and the host. The gut microbiota is highly diverse and dynamic, and its composition can be influenced by various factors. Another challenge is the safety and efficacy of targeting the gut microbiota and its metabolites. Although there is growing evidence suggesting that targeting the gut microbiota and its metabolites can have beneficial effects on T2DM, there is also the potential for unintended consequences. In addition, better methods are needed to assess the gut microbiota and its metabolites. Current methods for assessing the gut microbiota and its metabolites, such as 16s rRNA sequencing, metagenomics and chromatography-mass spectrometry, have limitations in terms of resolution and accuracy. Finally, more high-quality clinical trials with larger sample size are needed to verify their safety and efficacy on T2DM. Taken together, a comprehensive understanding the interaction between microbiota-derived metabolites and T2DM will shed light into potential targets for T2DM therapy.

## Author contributions

Conceptualization and design: MW and QX. Writing-original draft preparation: JW and KY. Writing-review and editing: JW, KY and HF. Project administration: JW. Funding acquisition: MW and QX. Manuscript revised: MW and QX. All authors have read and agreed to the published version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1114424/full#supplementary-material

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# Gene–diet interaction analysis using novel weighted food scores discovers the adipocytokine signaling pathway associated with the development of type 2 diabetes

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**Introduction:** The influence of dietary patterns measured using Recommended Food Score (RFS) with foods with high amounts of antioxidant nutrients for Type 2 diabetes (T2D) was analyzed. Our analysis aims to find associations between dietary patterns and T2D and conduct a gene-diet interaction analysis related to T2D.

**Methods:** Data analyzed in the current study were obtained from the Korean Genome and Epidemiology Study Cohort. The dietary patterns of 46 food items were assessed using a validated food frequency questionnaire. To maximize the predictive power of the RFS, we propose two weighted food scores, namely HisCoM-RFS calculated using the novel Hierarchical Structural Component model (HisCoM) and PLSDA-RFS calculated using Partial Least Squares-Discriminant Analysis (PLS-DA) method.

**Results:** Both RFS (OR: 1.11; 95% CI: 1.03- 1.20; P = 0.009) and PLSDA-RFS (OR: 1.10; 95% CI: 1.02-1.19, P = 0.011) were positively associated with T2D. Mapping of SNPs (P < 0.05) from the interaction analysis between SNPs and the food scores to genes and pathways yielded some 12 genes (CACNA2D3, RELN, DOCK2, SLIT3, CTNNA2, etc.) and pathways associated with T2D. The strongest association was observed with the adipocytokine signalling pathway, highlighting 32 genes (STAT3, MAPK10, MAPK8, IRS1, AKT1-3, ADIPOR2, etc.) most likely associated with T2D. Finally, the group of the subjects in low, intermediate and high using both the food scores and a polygenic risk score

found an association between diet quality groups with issues at high genetic risk of T2D.

**Conclusion:** A dietary pattern of poor amounts of antioxidant nutrients is associated with the risk of T2D, and diet affects pathway mechanisms involved in developing T2D.

KEYWORDS

type 2 diabetes, recommended food score, polygenic risk scores, case-control study, dietary patterns

# 1 Introduction

Diabetes is one of the most significant global public health concerns, imposing a heavy global burden on public health and socioeconomic development. Although incidence has started to decrease in some countries, the prevalence of diabetes has increased in recent decades in other developed and developing countries (1). Type 2 diabetes (T2D) makes up around 90% of cases of diabetes (2), and according to the World Health Organization, the number of people diagnosed with T2D is on the rise annually, even among young people (2).

The development of T2D is caused mainly by an interplay of unhealthy lifestyles and environmental and genetic factors. While some of these factors are under individual control, such as lifestyle, others are not, such as increasing age, sex, and genetics. Diet has also been firmly attributed to the risk of T2D (3, 4). This association has been confirmed in many prospective studies (5–8). In addition, T2D is an increasingly prevalent metabolic disorder causing severe micro- and macrovascular complications, namely, cardiovascular disease (CVD), retinopathy, neuropathy, and nephropathy (3, 9). Moreover, the beneficial effects of weight loss or lifestyle modification have been reported to prevent, delay, and reduce disease incidence (2, 10).

Therefore, valid estimation of overall dietary patterns (habitual food and nutrient intakes) has become a fundamental aspect of studying the relationships between diet and health status (8). General dietary habits can provide insights beyond the role of nutrients and single foods (2, 11). Some of the indices are based on national nutrition recommendations and national dietary guidelines that assess overall nutritional patterns, including the healthy eating index, alternate healthy eating index, healthy diet indicator, Recommended Food Score (RFS), diet quality index, Diet Quality Score, Mediterranean Diet Score (MDS), and Alternate Mediterranean Diet Score (aMDS). The RFS, MDS, and aMDS based on foods and food groups are relatively more straightforward in assessing overall dietary patterns and are based on food groups and nutrients (9, 11).

Recently, the pathophysiological influence of gene–lifestyle or gene–environment (G × E) interactions on the risk of T2D is currently under intensive research. Evidence of G × E interactions on the risk of development of T2D has been reported in many prospective studies reviewed here (3, 4). Here, G × E interaction analyses focusing on gene–diet interaction using RFS and SNPs while controlling for other confounding lifestyle factors like smoking, alcohol and coffee consumption, income and education levels, and so forth, were carried out for the Korean adult population. Odds ratios (ORs) with 95% confidence intervals (CIs) for the association and interaction analyses were calculated. Furthermore, the subjects were grouped into low, intermediate, and high diet quality groups using the food scores and genetic risk groups using an estimated global polygenic risk score (PRS), and interaction analyses between the groups were performed. Data from the Korean Genome and Epidemiology Study (KoGES) consortium, a prospective cohort study conducted in Korea in 2021, was used for our analysis (12–14).

However, a previous study using RFS for the Korean population could not show an acceptable association with the risk of T2D (11, 15, 16). This may be because the contributing power of each food item is different from each other: some food items contribute more than others. Therefore, weighted food scores were developed to maximize the unweighted RFS's interaction and predictive power. One score, HisCoM-RFS, was proposed using a novel statistical model called the Hierarchical Structural Component model (HisCoM). HisCoM estimates the weights for each food item used in the RFS calculation. HisCoM-RFS was contrasted for comparable results in different association analyses with PLSDA-RFS, another weighted food score calculated using the known partial least squares-discriminant analysis (PLS-DA) method. It finds another set of weights for each food item without considering food group categories. Both approaches assume a linear relationship exists between food items and the outcome T2D.

**Abbreviations:** KoGES, Korean Genome Epidemiology Study; METs, metabolic equivalents; RFS, Recommended Food Score; BMI, body mass index; GRG, genetic risk groups; DQG, diet quality groups; HisCoM, Hierarchical Structural Component model; PLS-DA, partial least squares-discriminant analysis method; HisCoM-RFS, weighted RFS calculated by HisCoM model; PLSDA-RFS, weighted RFS calculated by PLS-DA method; CI, confidence intervals.

# 2 Materials and methods

## 2.1 Study population

The study participants were recruited through the Korean Genome and Epidemiology Study (KoGES), a consortium

established for the identification of gene–environment factors and their interactions in commonly known diseases, such as T2D, hypertension, metabolic syndrome, obesity, and cardiovascular disease in Koreans (12). KoGES is a project comprising six prospective cohort studies categorized into population-based and gene–environment model studies extensively explained elsewhere (13, 14). We focused on the KoGES Ansan–Ansung study cohort whose data collection was initiated in 2001–2002 (baseline), with follow-up examinations conducted every 2 years. The participants were unrelated Korean individuals (N = 10,038) aged 40–69 years, representing urban (Ansan) and countryside (Ansung) populations. Our analyses involved data from the baseline recruit (17). Among the KoGES cohorts, the KoGES Ansan–Ansung cohort was chosen because it possesses the Frequency Food Questionnaire and has a more extended follow-up period than other cohorts.

## 2.2 Genotype data

The genotype data of the above participants were obtained through the Korea Association Resource (KARE) project, which was established in 2007 to conduct a large-scale genome-wide association study (GWAS) of the participants recruited through the KoGES Ansan–Ansung cohorts (18). The participants' common standard variant genotype data were generated using the Affymetrix Genome-Wide Human SNP array 5.0. The chip comprised around 50 million autosomal single-nucleotide polymorphisms (SNPs). There were 352,228 SNPs in 8,840 individuals left after quality control (QC) analysis. SNPs having minor allele frequencies<0.05, genotype calling rates<95%, and Hardy–Weinberg equilibrium P-values<$10^{-6}$ were removed. Only participants with consistent sex and calling rates (>90%) were preserved. Missing values of existing variants were imputed after QC, and PLINK (v1.90) (19) was used during QC. The SNPs were mapped to the UCSC hg19 genomic coordination. Missing genotype data were imputed using the Beagle 5.0 (20) software program.

## 2.3 Diagnosis of T2D subjects

After participants had fasted for at least 8 h, fasting plasma glucose (FPG; mg/dL), fasting plasma insulin (FPI; IU/mL), and triglycerides (TG; mg/dL) were measured. High-performance liquid chromatography was used to measure glycosylated hemoglobin (HbA1c). The following criteria were used to determine T2D subjects: (1) taking medication any for T2D; (2) fasting plasma glucose (FPG) ≥126 mg/dL, 2-h postprandial blood glucose (Glu120) ≥200 mg/dL, or glycated hemoglobin (HbA1c) ≥6.5%; and (3) age of disease onset ≥40 years. The following criteria selected normal subjects: (1) FPG<100 mg/dL, Glu120<140 mg/dL, and HbA1c<5.7% and (2) no history of diabetes (never been diagnosed with T2D) (21, 22). If a subject does not meet these criteria, then the subject is excluded from being a normal subject.

## 2.4 Covariates

We selected 10 covariates as adjustment and lifestyle factors for control during the analysis. This included age, sex, area (urban or village), body mass index (BMI), smoking, alcohol consumption, coffee consumption, metabolite equivalents (physical activity), education level, and income level. The covariates were assessed using self-administered questionnaires. The monthly household income is categorized into eight groups (0.5, 0.5~1, 1~1.5, 1.5~2, 2~3, 3~4, 4~6, and >6 million Korean won). Here 1,000 Korean won approximately corresponded to 0.9 US dollars. Smoking was categorized into non-smokers as well as former, occasional, and habitual smokers. Alcohol consumption was categorized into non-drinkers, former drinkers, and current drinkers. Time spent during five physical activity states (inactive, very light, light, moderate, and intense) were classified into nine ranges (0; none, 1;<30 min, 2; 30~60 min, 3; 60~90 min, 4; 90~2 h, 5; 2~3 h, 6; 3~4 h, 7; 4~5 h, 8; >5 h). These were converted to metabolic equivalents (METs) according to (17) (1.0 for inactive, 1.5 for very light, 2.4 for light, 5.0 for moderate, and 7.5 for intensive). The BMI (kg/$m^2$) of the participants was computed by dividing the weight (nearest 0.1 kg) in kilograms by the height (measured to the nearest 0.1 cm) in square meters. A further detailed description of the characteristics of the KoGES cohort can be found here (23). The list of the covariates used in our analyses is shown in Supplementary Table 1.

## 2.5 Dietary assessment

Dietary assessment was done through a validated semiquantitative food frequency questionnaire (FFQ) (24, 25), which records the consumption frequencies and portion sizes of 106 (Ansan and Ansung study) food items and drinks consumed during the previous year. The FFQ consisted of nine categories: never or seldom, once a month, one to two times a week, two to three times a week, three to four times a week, five to six times a week, once daily, twice daily, or more than three times daily. Furthermore, their daily frequency of meals was recorded as one meal a day, two meals a day, three meals a day, more than four meals a day, or irregular.

## 2.6 Recommended food score

Intake information from the FFQ was used to calculate the study subjects' RFS. RFS measures the overall dietary pattern of the individuals, a food tally based on reported consumption of foods bearing high amounts of antioxidant nutrients, consistent with the current American dietary guidelines of Kant et al., modified for the Korean population (11, 15, 16). A total of 45 food items (10 food groups) and one response for "daily frequency of meals" was selected and used to calculate the RFS score. Participants were assigned one point for each recommended food and regular eating

pattern (three meals a day) if they ate it at least once a week or more. The food items (and their corresponding points) for the RFS were as follows; daily frequency of meals (1), grains (1), legumes (4), vegetables (16), seaweeds (2), fruits/juices (12), fish (5), dairy products (3), nuts (1), and tea (1). Then, the score ranged from 0 to 46 points, and a higher score implies a better diet quality. The food items and their corresponding points for the RFS are shown in Supplementary Table 2. Subjects with increased consumption of foods rich in high antioxidant nutrients were given a higher score and lower scores to issues with lower consumption. All these antioxidant foods are healthy, and bad/unhealthy foods like sugar or sweets were not considered in the construction of this RFS.

## 2.7 HisCoM-RFS based on the HisCoM model

The calculated RFS assumes that each food item in a given category contributes equally to the diet quality of an individual. However, it is more reasonable to think that some food items contribute more than others. Therefore, we calculated a weighted food score using the RFS called HisCoM-RFS (Hierarchical Structural Component model (HisCoM) to analyze food scores) to capture this information. HisCoM estimates each food item's weight and the significance level between the food category and the outcome T2D. The HisCoM used here (Figure 1A) is a modification of the Pathway-based approach using HierArchical components of collapsed RAre variants Of the High-throughput sequencing data (PHARAOH) model (26) that was developed by our laboratory. PHARAOH employs ridge penalization to control for any correlations between variables. It assumes that the biomarkers have a linear relationship with a phenotype of interest while analyzing entire pathways simultaneously.

For HisCoM, let $y_i$ define a phenotype (T2D) of the $i^{th}$ $(i = 1, ., N)$ subject and assume that it independently follows an exponential family distribution. Let $T_k$ be the number of food items in the $k^{th}$ food category. Let $g_{itk}$ denote the food score of the $t^{th}$ item in the $k^{th}$ food category for the $i^{th}$ subject. Let $w_{itk}$ denote a weight assigned to $g_{itk}$ and $\beta_k$ indicate the coefficient connecting the $k^{th}$ food category to the phenotype. Specifically, the relationship between the food scores of each food item and the case–control phenotype is established in such a way that;

$$logit(\pi_i) = \beta_0 + \sum_{k=1}^{K}[\sum_{t=1}^{T_k} g_{itk} w_{itk}]\beta_k$$

Therefore, HisCoM-RFS is calculated as follows:

$$\text{HisCoM} - \text{RFS} = \sum_{k=1}^{K}[\sum_{t=1}^{T_k} g_{itk} w_{itk}]\beta_k$$

where HisCoM estimated w and β . The $logit(\pi_i)$ is the logit function from logistic regression models explaining the log of odds (ratio of T2D subjects to normal subjects).

To estimate parameters HisCoM, the alternating least squares (ALS) algorithm was used, which was initially proposed by de Leeuw et al. (27) and adopted for the generalized structured component analysis (GSCA) (28) and later for the penalized log-likelihood function (26). PHARAOH employed the ALS algorithm in its penalized log-likelihood part (26). This ALS algorithm consists of two steps that iterate until convergence;

*Step 1*: For fixing the weight coefficient estimates $w_{itk}$ , update the food category coefficient estimates $\beta_k$ , in the sense of least squares.

*Step 2*: For fixing food category coefficient estimates $\beta_k$ , update the weight coefficient estimates $w_{itk}$ , in the sense of least squares.

We use a penalization approach to consider potential correlations between food items and categories. In this study, we adopt a ridge-type penalty to control multicollinearity between food items ($\lambda_1$) only and not between food categories ($\lambda_2 = 0$) so that the phenotype is a linear combination of the food items and not the food categories. The significance of the estimated parameters was tested through the permutation by resampling the phenotypes.

## 2.8 PLSDA-RFS based on the PLS-DA method

In addition to the HisCoM method, another weighted food score called PLSDA-RFS was calculated using the commonly known partial least squares regression (PLS-R) for discriminant analysis (PLS-DA) method (29). PLS-DA is derived from PLS-R, where the response vector assumes discrete values (T2D) and considers the correlation between T2D and the food items while maximizing the covariance between T2D and the weights calculated (30, 31). PLS-DA incorporates T2D and RFS information in defining the scores and loadings (weights) used to calculate PLSDA-RFS. However, PLS-DA does not consider the food groups during the weight and coefficient calculation. PLSDA-RFS was calculated by multiplying the previously calculated unweighted RFS scores with the estimated weight matrix and the coefficient values in the first column of the estimated coefficient matrix.

## 2.9 Statistical analyses

Unless specified, statistical analyses were conducted using R software (version 4.2.1) to identify the association between T2D and diet. Categorical and continuous variables for participants' general characteristics according to the case–control study for T2D were compared using the chi-squared test ($\chi^2$test) and two-sample t-test, respectively. The generalized linear regression model (GLM) was used to find the association ORs (95% CI) between diet (RFS, HisCoM-RFS, and PLSDA-RFS) and T2D. Secondly, the food scores were grouped into low, intermediate, and high diet quality groups and their ORs (95% CI) were estimated. After ranking the food scores, all food scores with ranks below 33.33% were grouped as low, intermediate for those below 66.6%, and above 66.6% as high. Thirdly, since genetic and lifestyle factors influence the development of T2D, gene–diet interaction analysis focused on the "interaction effect," unlike the "main effect" between SNPs and food scores, was performed to identify SNPs, genes, and pathways associated with T2D. A significant interaction shows the role of dietary habits affecting pathways during the development of T2D. Logistic regression in PLINK (v1.90, Windows) was used for this analysis (19, 32). The analyses were adjusted for age, sex, area, BMI,

smoking, alcohol consumption, coffee consumption, education level, income level, and METs following other studies involving KoGES Ansan–Ansung data (2, 16, 33–37). A statistical significance level of $P< 0.05$ was used unless specified. To find genes and pathways, significant SNPs from the interaction effect were mapped to genes and then pathways using the Multi-marker Analysis of GenoMic Annotation (MAGMA, windows version) tool, a generalized gene-set analysis tool of GWAS data (38, 39). MAGMA analyzes genes and pathways by multiple linear regression after principal component analysis for each gene. Pathway information was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (40) database, whereas the gene location file (GRCh37) was downloaded from the National Center for Biotechnology Information (NCBI) website. Lastly, a global polygenic risk score (PRS) for T2D was generated using independent summary statistics (N = 191,764; 36,614 cases and 155,150 controls) from Biobank Japan (Supplementary Figure 1) (41). LDpred (42) was used to reweight each variant according to (1) the effect size, (2) the strength of statistical significance observed for T2D, and (3) linkage disequilibrium (LD) between a variant and others nearby. A tuning parameter that denotes the proportion of causal variants (P) estimated with the validation samples (P = 0.1) was selected. Nine categories capturing the interactions between genetic risk (low (reference), intermediate, and high) based on PRS and diet quality (low, intermediate, and high (reference)) based on the food scores were created. Adjusted ORs of the nine categories were calculated.

# 3 Results

## 3.1 Baseline characteristics of the subjects

A total of 350,000 SNPs and 8,840 subjects were left after genotype data QC. Diagnosis of the subjects for T2D left us with 4,975 subjects (1,288 cases and 3,687 control). The control subjects here are normal subjects without T2D. After adjusting for the covariates (for age, sex, area, BMI, smoking, alcohol consumption, coffee consumption, education levels, income levels, and METs), 4,292 (1,090 cases and 3,202 control) subjects were left. The characteristics of the 4,292 subjects are summarized in Table 1, presented as means ± standard deviation for continuous variables and percentage proportions for categorical variables. Smoking and alcohol consumption were left with two groups after data preprocessing. Income was summarized into five levels (<0.5~1, 1.0~2.0, 2.0~3.0, 3.0~4.0, >4.0), education into four classes (combined college, university, and graduate school into one level of higher education) and coffee consumption into four groups (never/seldom, monthly, weekly, and daily) in all analyses, to reduce on the number of levels of these variables. T2D was significantly associated with the area, sex, age, BMI, smoking, education level, income level, coffee consumption, PLSDA-RFS, and PRS.

## 3.2 HisCoM-RFS and PLSDA-RFS

The HisCoM and PLS-DA methods estimated the weights and coefficients ($\beta$)used to calculate the weighted food scores HisCoM-RFS and PLSDA-RFS, as shown in Figure 1A. HisCoM estimated the weights of the 45 food items, the daily frequency of meals, and the coefficients of the 10 food categories and is shown in Supplementary Tables 2, 3. PLS-DA also estimated the coefficients and weights of the 45 food items and the daily frequency of meals and was used to calculate PLSDA-RFS. The density plots of RFS, HisCoM-RFS, and PLSDA-RFS for control subjects (yellow; n = 3,347; bandwidth = 1.351, 0.04067 and 0.002164, respectively) and T2D subjects (green, n = 1,159; bandwidth = 1.653, 0.04929 and 0.002787, respectively) are shown in Figure 1B. There are no noticeable differences between the density plots between T2D and control subjects but between the food scores. We compared the

TABLE 1 Baseline characteristics and food scores of participants according to T2D case–control subjects.

| T2D case–control study | | | |
|---|---|---|---|
| | Case (n = 1,090) | Control (n = 3,202) | *P-value* |
| *Area* | | | 3.6E-07 |
| Ansung | 511 (46.88) | 1,219 (38.07) | |
| Ansan | 579 (53.12) | 1,983 (61.93) | |
| *Sex* | | | 3.8E-06 |
| Male | 589 (54.04) | 1,469 (45.88) | |
| Female | 501 (45.96) | 1,733 (54.12) | |
| *Age* | 55.77 ( ± 8.76) | 49.65 (8.26) | < 2.2E-16 |
| *BMI ($kg/m^2$)* | 25.59 (± 3.27) | 24.13 (± 2.89) | < 2.2E-16 |
| *METs* | 42.92 (± 24.84) | 42.00 (± 23.97) | 0.284 |
| *Alcohol consumption* | | | 0.077 |

*(Continued)*

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TABLE 1 Continued

| T2D case–control study | | | |
|---|---|---|---|
| | Case (n = 1,090) | Control (n = 3,202) | *P-value* |
| Non-drinkers | 590 (54.13) | 1,632 (50.97) | |
| Former drinkers | 500 (45.87) | 1,570 (49.03) | |
| Current drinkers | 0 (0) | 0 (0) | |
| *Smoking* | | | 0.033 |
| Never (non-smoker) | 0 (0) | 0 (0) | |
| Former smoker | 829 (76.06) | 253 (79.20) | |
| Occasional smoker | 261 (23.94) | 666 (20. 80) | |
| Habitual smoker | 0 (0) | 0 (0) | |
| *Education level* | | | < 2.2E-16 |
| ≤Elementary school | 444 (40.73) | 817 (25.52) | |
| Middle school | 221 (20.28) | 726 (22.67) | |
| High school | 286 (26.24) | 1,159 (36. 20) | |
| College | 28 (2.57) | 139 (4.34) | |
| University | 99 (9.08) | 301 (9.40) | |
| Graduate school (higher) | 12 (1.10) | 60 (1.87) | |
| *Income level (million Won)* | | | < 2.2E−16 |
| <0.5 | 271 (24.86) | 426 (13.30) | |
| 0.5~1 | 172 (15.78) | 428 (13.37) | |
| 1.0~1.5 | 158 (14.50) | 511 (15.96) | |
| 1.5~2.0 | 142 (13.03) | 465 (14. 52) | |
| 2.0~3.0 | 160 (14.68) | 690 (21.55) | |
| 3.0~4.0 | 96 (8.81) | 407 (12.71) | |
| 4.0~6.0 | 61 (5.60) | 199 (6.21) | |
| >6.0 | 30 (2.75) | 76 (2.37) | |
| *Coffee consumption frequency* | | | 0.0006 |
| Never or seldom | 299 (27.43) | 686 (21.42) | |
| Once a month | 35 (3.21) | 99 (3.09) | |
| 1–2 weeks | 36 (3.30) | 83 (2.59) | |
| 2–3 weeks | 79 (7.25) | 232 (7.25) | |
| 3–4 weeks | 65 (5.96) | 228 (7.12) | |
| 5–6 weeks | 25 (2.29) | 104 (3.25) | |
| One daily | 321 (29.45) | 926 (28.92) | |
| Twice daily | 108 (9.91) | 406 (12.68) | |
| >3 daily | 122 (11.19) | 438 (13.68) | |
| Recommended Food Scores | | | |
| RFS | 17.02 (7.53) | 17.18 (7.56) | 0.545 |
| HisCoM-RFS | −0.16 (0.23) | −0.16 (0.23) | 0.828 |
| PLSDA-RFS | 0.02 (0.01) | 0.02 (0.01) | 0.023 |
| PRS | −1.54 (0.20) | −1.65 (0.20) | <2.2E−16 |

N = 4,292; values are n (%) for categorical variables and mean ± SD for continuous variables. Differences in characteristics were analyzed using $\chi^2$ tests for categorical variables and two-sample t-tests for continuous variables. METs, metabolic equivalents; RFS, Recommended Food Scores; PRS, polygenic risk scores.

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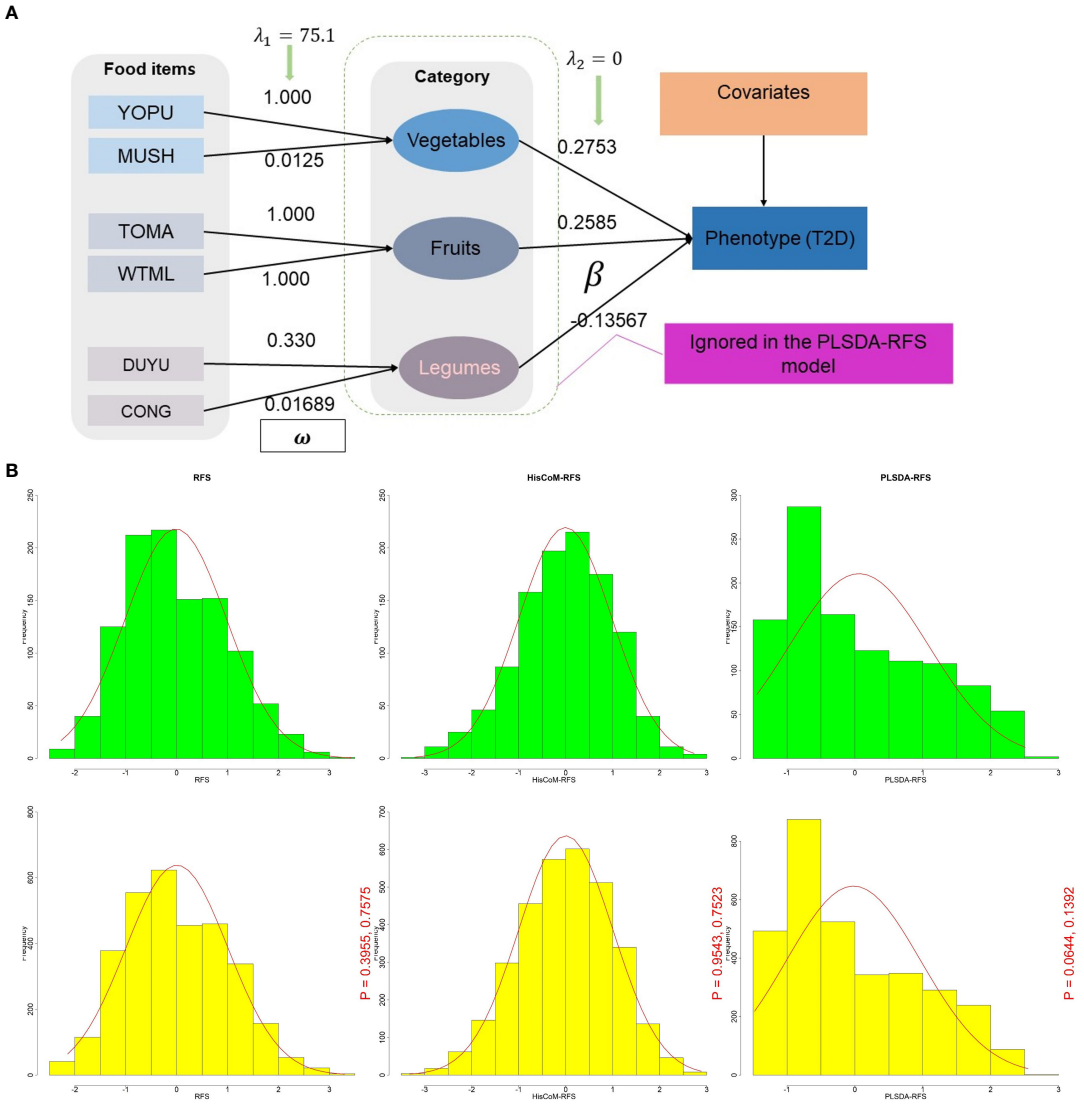


FIGURE 1
**(A)** A schematic diagram of the HisCoM model showing HisCoM-RFS calculation using three food categories. The rectangles and ellipses represent food items and food categories, respectively. So that the phenotype is a linear combination of the food items and not the food categories. HisCoM, Hierarchical Structural Component model. **(B)** Density plot distribution of RFS, HisCoM-RFS, and PLSDA-RFS between T2D (green) and control subjects (yellow). The p-values are from the Wilcoxon rank sum test and the Kolmogorov-Smirnov test, respectively between green and yellow. RFS, Recommended Food Scores; HisCoM, Hierarchical Structural Component model; PLS-DA, Partial Least Squares-D-iscriminant Analysis method; HisCoM-RFS, weighted RFS calculated by HisCoM model; PLSDA-RFS, weighted RFS calculated by PLS-DA method.

distribution of the food scores between case and control subjects using the Wilcoxon rank-sum test and the Kolmogorov–Smirnov test. The P-values of the tests are shown in Figure 1B. The Wilcoxon rank-sum test showed that the two groups are not different, whereas the Kolmogorov–Smirnov test showed that the two groups come from the same distribution.

## 3.3 Association between diet quality measured using food scores and T2D

Of the three food scores, only PLS-DA was positively associated with T2D unadjusted for the other covariates (OR: 1.0839; 95% CI: 0.9293–1.0622; $P = 0.0203$). The food scores were standardized for comparable results. After adjusting for potential covariates (age, sex, BMI, and area) and lifestyle factors (smoking, alcohol and coffee consumption, education level, income level, and METs), both RFS (OR: 1.11; 95% CI: 1.03–1.20; $P = 0.014$) and PLSDA-RFS (OR: 1.10; 95% CI: 1.02–1.19, $P = 0.011$) were positively associated with T2D, as shown in Figure 2. This indicates that a person's dietary patterns can affect the development of T2D. Grouping the food scores into low, intermediate, and high diet quality groups, with high being the reference group, showed the low diet quality group of RFS (OR: 0.83; 95% CI: 0.68–1.01, $P = 0.059$) and the intermediate diet quality group of PLSDA-RFS (OR: 0.80, 95% CI: 0.73–1.06; $P = 0.017$) to be associated with T2D, as shown in Figure 3. From the two analyses,

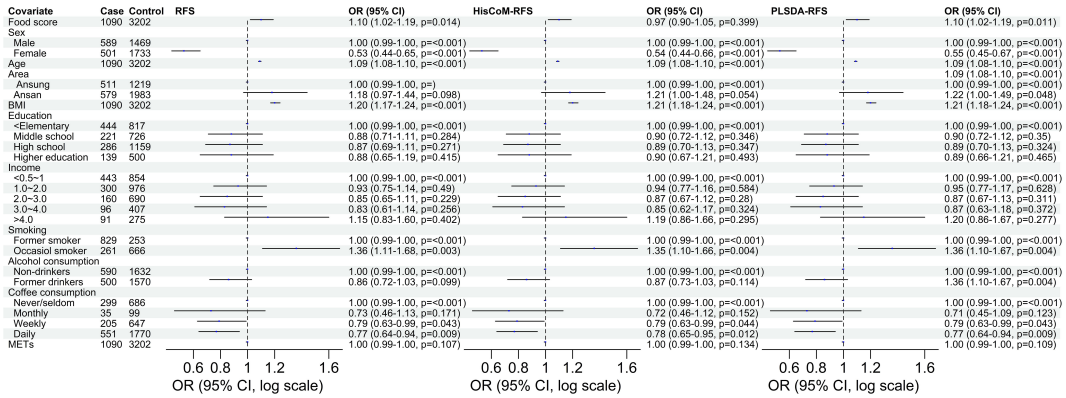


FIGURE 2
The odd ratios of RFS, HisCoM-RFS and PLSDA-RFS adjusted for age, sex, area, BMI, income level, education level, smoking, alcohol and coffee consumption, and METs, showing the association between dietary habits and T2D. The *P*-values were calculated using multiple logistic regression. METs, metabolic equivalents; BMI, body mass index; RFS, recommended food scores.

being female, age, BMI, being an occasional smoker, and at least weekly and daily consumption of coffee were constantly associated with T2D ($P< 0.05$).

## 3.4 Gene–diet interaction analysis

The 8,205, 9,331, and 103,408 SNPs for RFS, HisCoM-RFS, and PLSDA-RFS, respectively (3,301, 59, and 4,260 SNPs were significant at $P< 0.001$), were significant with "interaction effect" $P< 0.05$. Their Manhattan plots are shown in Supplementary Figure 1. These SNPs were mapped to genes and pathways using the bioinformatics tool MAGMA. Table 2 shows the 19 and 29 genes (12 common genes) found in the gene analysis step of MAGMA using RFS and PLSDA-RFS, respectively, at $P< 1.0E-08$. HisCoM-RFS did not yield any significant genes at $P< 1.0E-08$. The 12 genes common to both RFS and PLSDA-RFS include FHIT, CACNA2D3, ITPR1, RELN, CNTNAP2, CTNNA2, DOCK2, ROBO2, SLIT3, MAG12, ASIC2, and CREB5. Supplementary Table 4 shows the pathways from the pathway analysis (or gene-set analysis) step of MAGMA for RFS, HisCoM-RFS, and PLSDA-RFS at $P< 0.05$. Pathway analysis found some of the pathways to be associated with T2D in literature, for example, pathways such as the insulin signaling pathway, adipocytokine signaling pathway, type II diabetes, prostate cancer, and other metabolic pathways (43–49). Multiple comparison corrections of the discovered pathways using the false discovery rate (FDR) correction method found vascular smooth muscle contraction (q-value = 0.06), small cell lung cancer (q-value = 0.007), long-term potentiation (q-value = 0.065), and adipocytokine signaling (q-value = 0.026) pathways (FDR q-value< 0.1). The strongest association was observed with the adipocytokine signaling pathway yielding a significant gene set of 32 genes listed in Table 3. Some genes include STAT3, AKT1-AKT3, MAPK10, MAPK8, PRKAA1, ACSL1, CAMKK1 RXRG, and NFKB. Finally, genetic risk assessed using global PRS showed a strong positive association with T2D adjusted for the covariates (OR: 17.78; 95%

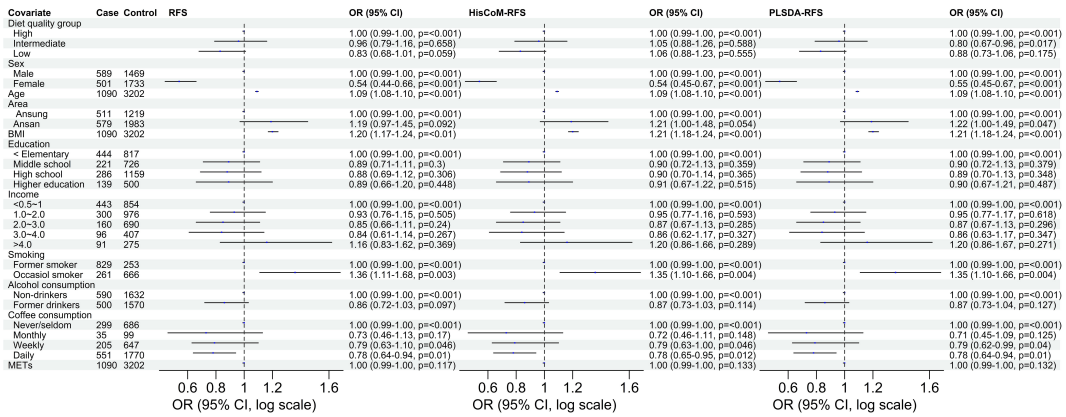


FIGURE 3
The odd ratios of RFS, HisCoM-RFS and PLSDA-RFS diet quality groups adjusted for age, sex, area, BMI, income level, education level, smoking, alcohol and coffee consumption, and METs, showing the association between dietary quality and T2D. High diet quality group is the reference group. The *P*-values were calculated using multiple logistic regression. METs, metabolic equivalents; BMI, body mass index; RFS, recommended food scores.

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TABLE 2 List of significant genes from the gene analysis step of MAGMA for RFS and PLSDA-RFS.

| RFS | | | | PLSDA-RFS | | | |
|---|---|---|---|---|---|---|---|
| GENE | CHROMOSOME | No. of SNPs | *P* | GENE | CHROMOSOME | No. of SNPs | P |
| ASIC2 | 17 | 78 | 6.48E−14 | ASIC2 | 17 | 111 | 1.13E−13 |
| CACNA2D3 | 3 | 59 | 6.58E−09 | CACNA1C | 12 | 34 | 3.17E−11 |
| CREB5 | 7 | 97 | 1.07E−10 | CACNA2D3 | 3 | 75 | 1.40E−11 |
| CTNNA2 | 7 | 48 | 3.01E−11 | CACNB3 | 10 | 49 | 2.44E−09 |
| DOCK2 | 2 | 56 | 2.38E−10 | CDH4 | 20 | 43 | 1.73E−09 |
| ERBB4 | 5 | 45 | 1.07E−10 | CNTNAP2 | 7 | 124 | 1.89E−11 |
| FGF12 | 2 | 34 | 3.29E−10 | CREB5 | 7 | 39 | 6.5E−10 |
| FHIT | 3 | 32 | 3.56E−09 | CTNNA2 | 2 | 50 | 1.56E−11 |
| GABRG3 | 3 | 94 | 1.51E−11 | CTNNA3 | 10 | 92 | 2.95E−12 |
| GRM7 | 15 | 17 | 4.13E−09 | DOCK2 | 5 | 30 | 1.36E−09 |
| ITPR1 | 3 | 67 | 3.75E−10 | FGF14 | 13 | 45 | 7.60E−11 |
| KCNMA1 | 3 | 27 | 1.29E−09 | FHIT | 3 | 92 | 6.51E−11 |
| MAGI2 | 10 | 45 | 2.88E−10 | FMN2 | 1 | 34 | 3.05E−09 |
| MAGI2 | 7 | 83 | 1.28E−10 | GALNT18 | 11 | 26 | 2.86E−09 |
| PDE4D | 5 | 50 | 8.41E−09 | ITRP1 | 3 | 26 | 8.30E−09 |
| RELN | 7 | 39 | 1.06E−09 | MAGI2 | 7 | 67 | 6.97E−12 |
| ROBO2 | 3 | 95 | 8.53E−12 | NRXN1 | 2 | 66 | 5.39E−09 |
| RYR3 | 15 | 28 | 2.05E−09 | NRXN3 | 14 | 64 | 1.47E−09 |
| SLIT3 | 3 | 58 | 3.62E−09 | PRKCA | 17 | 33 | 2.04E−09 |
| | | | | PRKCE | 2 | 31 | 3.28E−11 |
| | | | | PRKG1 | 10 | 65 | 5.20E−10 |
| | | | | PSD3 | 8 | 42 | 2.35E−09 |
| | | | | PTPRN2 | 7 | 35 | 7.74E−09 |
| | | | | RELN | 7 | 38 | 7.72E−09 |
| | | | | ROBO2 | 3 | 71 | 2.13E−11 |
| | | | | RPS6KA2 | 6 | 46 | 5.93E−11 |
| | | | | RYR2 | 1 | 41 | 9.98E−11 |
| | | | | SLIT3 | 5 | 55 | 5.43E−11 |
| | | | | UST | 6 | 19 | 1.51E−09 |

CI: 12.01–26.50; $P< 0.01$). Grouping of the PRS into low, intermediate, and high genetic risk groups with the low genetic risk as the reference as shown in Supplementary Figure 3 showed both intermediate (OR: 1.46; 95% CI: 1.19–1.79; $P< 0.01$) and high (OR: 3.36; 95% CI: 2.77–4.08; $P< 0.01$) genetic risk groups having an association with T2D, as shown in Supplementary Figure 3. The nine groups showing interactions between diet quality groups and genetic risk groups found significant interactions between different genetic risk groups and diet quality groups, as shown in Figure 4, especially the high GRC and the diet quality groups.

# 4 Discussion

Investigating the role of dietary patterns in association with T2D is still a hot research topic worldwide. A previous study showed that a higher RFS score is associated with lower oxidative stress but failed to show an association with T2D in Korean adults (16). To maximize the interaction power of RFS on T2D, we developed weighted RFS scores HisCoM-RFS and PLSDA-RFS using the HisCoM and PLS-DA models, which determine the weights of the food items. The development of weighted food

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TABLE 3 List of genes from the gene set of the adipocytokine signaling pathway.

| Gene | Chromosome | No. of SNPs | *P* |
|---|---|---|---|
| ACACB | 12 | 9 | 0.0003 |
| ACSL1 | 4 | 4 | 0.0058 |
| ACSL3 | 2 | 5 | 0.0089 |
| ACSL5 | 10 | 5 | 0.0096 |
| ACSL6 | 5 | 5 | 0.007 |
| ADIPOR2 | 12 | 2 | 0.0042 |
| AKT1 | 14 | 2 | 0.0144 |
| AKT2 | 19 | 2 | 0.015 |
| AKT3 | 1 | 4 | 0.0135 |
| CAMKK1 | 17 | 1 | 0.0002 |
| CAMKK2 | 12 | 2 | 0.0106 |
| CD36 | 7 | 3 | 0.0004 |
| IKBKB | 8 | 1 | 0.0014 |
| IRS1 | 2 | 6 | 0.0031 |
| LEPR | 1 | 2 | 0.0167 |
| MAPK10 | 4 | 9 | 0.0033 |
| MAPK8 | 10 | 9 | 0.0014 |
| NFKB1 | 4 | 14 | 0.0022 |
| NPY | 7 | 1 | 0.0007 |
| POMC | 2 | 1 | 0.04 |
| PPARA | 22 | 1 | 0.012 |
| PPARGC1A | 4 | 35 | 1.32E-07 |
| PRKAA1 | 5 | 2 | 0.0332 |
| PRKAA2 | 1 | 2 | 0.0047 |
| PRKAG2 | 7 | 4 | 0.0006 |
| PRKCQ | 10 | 18 | 3.7E−05 |
| RXRA | 9 | 1 | 0.003 |
| RXRG | 1 | 2 | 0.0071 |
| SLC2A1 | 1 | 1 | 0.0059 |
| STAT3 | 17 | 4 | 0.0413 |
| TNFRSF1A | 12 | 1 | 0.0033 |

scores is based on the assumption that some food items contribute more than others to the overall food score given the phenotype. After calculating the weighted food scores, we performed analyses contrasting these food scores about the association of dietary patterns with the development of T2D while adjusting for covariates and other lifestyle factors like METs, smoking, alcohol and coffee consumption, and education and income levels. Firstly, a significant association was found between dietary habits, mainly with the weighted food score PLSDA-RFS, unlike the former unweighted RFS score in the previous study (16). After adjusting the 10 covariates, unweighted RFS and PLSDA-RFS food scores were significantly associated with T2D. Grouping the food scores into low, intermediate, and high diet quality groups showed intermediate and low diet quality groups to be associated with T2D. This shows the importance of high diet quality (foods rich in antioxidant properties) playing a preventive role in the occurrence of T2D.

In the literature, a higher Dietary Approaches to Stop Hypertension (DASH) Score was associated with lower T2D risk in men (50). An extended follow-up of urban Chinese adults showed that a higher healthy diet score (HDS) was associated with lower diabetes risk (51). Other studies also associated diet quality with the risk of T2D (9, 52). Secondly, interaction analysis between food scores and SNPs focusing on the "interaction effect" instead of the "main effect" aimed to find genes and pathways associated with T2D. Significant interaction implies that diet is involved with pathway mechanisms related to the development of T2D. The interaction analysis with the respective food scores RFS, HisCoM-RFS, and PLSDA-RFS yielded some significant SNPs ($P< 0.05$), filtered and used in MAGMA's gene and pathway analysis steps. We did not get any SNPs below the GWAS significance level of $P< 5.0E-08$. Gene analysis yielded 19 genes and 29 genes at $P< 1.0E-08$ with RFS and PLSDA-RFS, respectively, with 12 common genes, namely, FHIT, CACNA2D3, ITPR1, RELN, CNTNAP2, CTNNA2, DOCK2, ROBO2, SLIT3, MAGI2, ASIC2, and CREB5. FHIT is involved in purine metabolism, and CACNA2D3 is engaged with the voltage-dependent calcium channel. Calcium signaling is crucial for insulin secretion in pancreatic cells (53, 54). RELN gene encodes a large secreted extracellular matrix protein thought to control cell–cell interactions critical for cell positioning and neuronal migration during brain development and is involved in multiple disorders. CTNNA2 enables actin filament binding activity, whereas DOCK2 remodels the actin cytoskeleton required for lymphocyte migration in response to chemokine signaling. SLIT3 is associated with cell receptors during cellular migration (55).

The pathway analysis revealed many pathways, some of which have been associated with T2D in literature. The pathways are mainly related to cancer, metabolism, and signaling. However, FDR correction left vascular smooth muscle contraction ($q$-value = 0.06), small cell lung cancer ($q$-value = 0.007), long-term potentiation ($q$-value = 0.065), and adipocytokine signaling ($q$-value = 0.026) pathways to be strongly associated with T2D at q-value< 0.1. The strongest association was observed with the adipocytokine signaling pathway, which produced a gene set of 32 genes in this pathway strongly associated with T2D. These genes include STAT3, AKT1-AKT3, MAPK10, MAPK8, IRS1, ADIPOR2, ACSL1, CAMKK1, RXRG, and NFKB1. STAT3 is involved in cytokine- and nutrient-induced insulin resistance, and its phosphorylation contributes to skeletal muscle insulin resistance in T2D (56). MAPK10 was identified as a critical gene in diabetes mellitus-induced atrial fibrillation in mice (57). The AKT genes and IRS1 may influence adipocyte insulin resistance (58–61). Variants in the ADIPOR2 gene are associated with increased diabetic risk (62, 63). In a meta-analysis study, RXRG, NFKB1, ACSL1, and CAMKK1 genes were also associated with T2D (64). Briefly, insulin resistance is one of

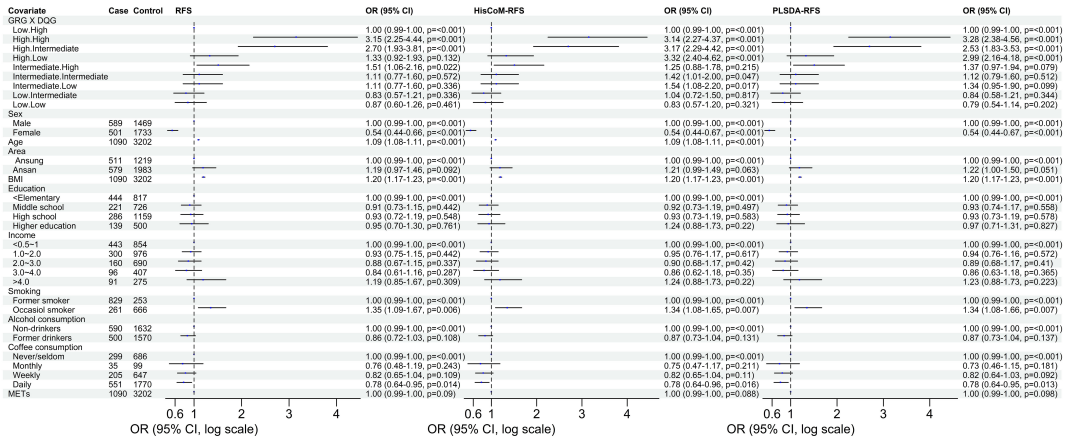


FIGURE 4
The odd ratios of the interaction between the genetic risk groups and diet quality groups of RFS, HisCoM-RFS and PLSDA-RFS. The analyses were adjusted for the ten covariates. Interaction of low GRC and high DQG is the reference group. METs, metabolic equivalents; BMI, body mass index; RFS, recommended food scores; GRG, genetic risk groups; DQG, diet quality groups.

the major hallmarks of the pathogenesis and etiology of T2D (48). It is reflected by impairments in insulin signaling in the diabetic state displaying a reduced insulin sensitivity (43). A generally accepted view is that insulin resistance associated with T2D is caused by defects at one or several levels of the insulin-signaling cascade, for example, in skeletal muscles, adipose tissue, and liver, that quantitatively constitute the bulk of the insulin-responsive tissues (45). Adipocytes and resident macrophages that have migrated to the adipose tissue produce and secrete adipocytokines, including tumor necrosis factor-α, interleukin-6, resistin, and adiponectin, which are thought to contribute to the development of insulin resistance and T2D (46, 47, 64). Dysregulation of vascular smooth muscle excitability using calcium ions occurs during T2D disorder (65–67). Abnormal long-term potentiation behavior is observed in patients with T2D (68, 69).

One limitation of the study is that the genotype data were not imputed with the 1000G population data when the analysis was carried out. Also, larger cohort data are needed to replicate these findings. In the future, we will perform the same analysis using genotype data imputed using 1000G and replicate the findings of our analysis using an independent dataset.

In conclusion, this study revealed the association between dietary patterns and the development of T2D. The risk of T2D increases in individuals with poor dietary habits (foods lacking antioxidant properties). Lifestyle habits like smoking, BMI, age, and alcohol and coffee consumption increase the risk of T2D. The impact of genetics was also observed, especially in people with high genetic risks. The interaction between diet and genetics showed that dietary patterns affect pathway mechanisms in the development of T2D. The study results elucidate the protective role of a healthy diet in lowering the risk of T2D. However, further prospective investigations, more rigorous studies of larger cohorts, intervention research, or different methods of constructing food (indices) quality scores will be needed to investigate if diet can predict the prevalence of T2D (causal–effect relationship). Also, further validation studies of the above pathways are required to find T2D biochemical pathogenesis conclusively.

## Data availability statement

The data analyzed in this study is subject to license/restrictions: Data supporting this paper were obtained from the National Biobank of Korea (NBK). The KoGES epidemiology data and the KARE genotype data are available only upon agreement with NBK. Requests to access these data can be directed to National Human Resources Bank (http://biobank.nih.go.kr 1661-9070) or the Human Biobank Information System (HuBIS desk) at https://is.kdca.go.kr/. BBJ summary statistics used in this study were downloaded from the Biobank Japan PheWeb: https://pheweb.jp/.

## Author contributions

The authors' responsibilities were as follows: conceptualization: TP. Methodology: TP and CA. Formal analysis: CA. Resources: TP and OK. Writing—original draft preparation: CA. Writing—review and editing: CA, TP, WC, and MM. Supervision: TP. Project administration: TP. Funding acquisition: TP. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1165744/full#supplementary-material

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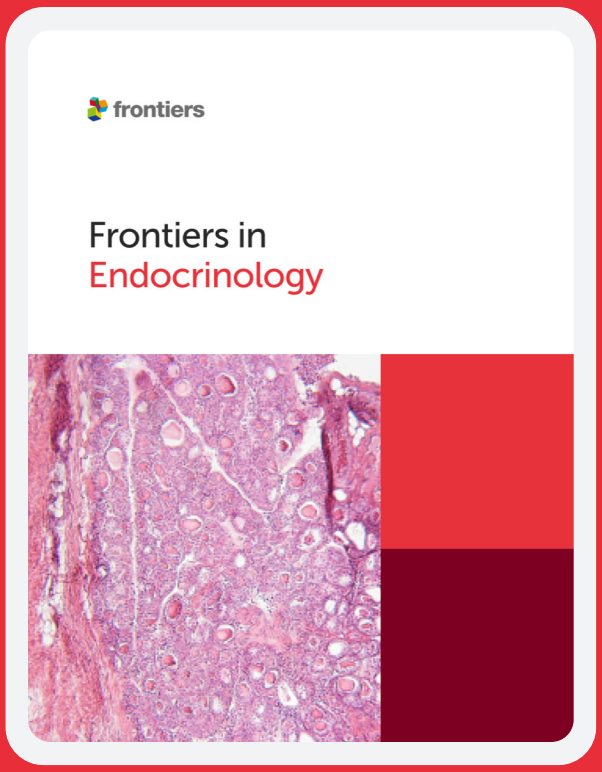


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