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The mechanism in gut microbiota of diabetes and endocrine complications: preventive and therapeutic target

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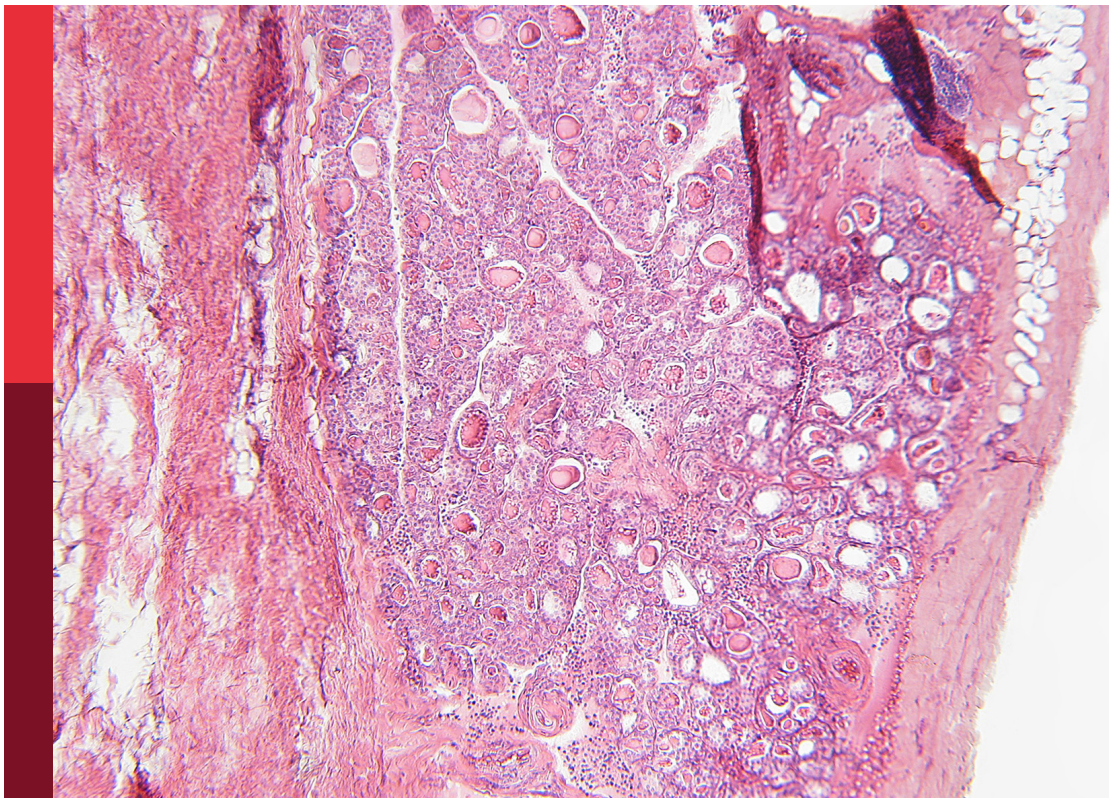

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The mechanism in gut microbiota of diabetes and endocrine complications: preventive and therapeutic target

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Association between gut microbiota and diabetic microvascular complications: a two-sample Mendelian randomization study

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Background: Gut microbiota (GM) homeostasis in the human body is closely associated with health, which can be used as a regulator for preventing the onset and progression of disease. Diabetic microvascular complications bring about not only a huge economic burden to society, but also miserable mental and physical pain. Thus, alteration of the GM may be a method to delay diabetic microvascular complications.

Objective: A two-sample Mendelian randomization (MR) analysis was conducted to reveal the causal inference between GM and three core diabetic microvascular complications, namely, diabetic kidney disease (DKD), diabetic retinopathy (DR), and diabetic neuropathy (DNP).

Methods: First, genome-wide association study (GWAS) summary statistics for GM from the MiBioGen consortium and three main diabetic microvascular complications acquired from the FinnGen research project were assessed. Second, a forward MR analysis was conducted to assess the causality of GM on the risk of DKD, DR, and DNP. Third, a series of sensitivity studies, such as heterogeneity tests, pleiotropy evaluations, and leave-one-out analyses, were further conducted to assess the accuracy of MR analysis. Finally, Steiger tests and reverse MR analyses were performed to appraise the possibility of reverse causation.

Results: A total of 2,092 single-nucleotide polymorphisms related to 196 bacterial traits were selected as instrumental variables. This two-sample MR analysis provided strongly reasonable evidence that 28 genetically predicted abundance of specific GM that played non-negligible roles in the occurrence of DKD, DR, and DNP complications were causally associated with 23 GM, the odds ratio of which generally ranged from 0.9 to 1.1. Further sensitivity analysis indicated low heterogeneity, low pleiotropy, and high reliability of the causal estimates.

Conclusion: The study raised the possibility that GM may be a potential target to prevent and delay the progression of diabetic microvascular complications. Further experiments of GM therapy on diabetic microvascular complications are warranted to clarify their effects and specific mechanisms.

KEYWORDS

diabetic kidney disease, diabetic microvascular complications, diabetic neuropathy, diabetic retinopathy, Mendelian randomization

Introduction

Diabetic microvascular complications are characterized by the damage of small vessels or nerves as a result of chronic persistent hyperglycemic state in patients with diabetes mellitus (DM), manifested as abnormal structure and changes in functions of the corresponding targeted organs ultimately (1–3). It is known that diabetic kidney disease (DKD), diabetic retinopathy (DR), and diabetic neuropathy (DNP) are three major diabetic chronic microvascular complications that need to be screened comprehensively upon diagnosis of type 2 diabetes (T2D) and type 1 diabetes (T1D) in the fifth year and even at least annually thereafter because of the characteristics of insidious onset and irreversible progression, which leads to enormous economic burden and prolonged potential physical and mental suffering (4, 5). In spite of the large number of novel treatments available, the incidence and prevalence of DM continue to increase around the world and show a trend of younger generations being affected, which leads to an obvious rise in the corresponding microvascular complications (6–8).

Plenty of microbes are enriched in the gastrointestinal tract, the biggest microbiota habitat in the human body; meanwhile, these microbiota exist in a dynamic balanced state for health regulation purposes (9). The composition and metabolism of gut microbiota (GM) play an important role in DM and its complications (10, 11), which is affected by multiple factors such as diet (12), demographics (13), and use of medication (14). Recent studies have focused on the association between GM and DM and its microvascular complications and especially put forward the theory of "gut–kidney axis" (15), "gut–retina axis" (16), "gut–brain axis" (17), and "gut–peripheral nerve axis" (18). Therefore, focusing on the modulation of GM with probiotics, prebiotics, synbiotics, or even fecal microbial transplantation may be a promising breakthrough direction on DM and subsequent microvascular complications. Nonetheless, the link between GM and diabetic microvascular complications driven by causative mechanistic interactions or merely being correlative remains unclear.

Mendelian randomization (MR) is a complementary statistical approach that leverages the genetic variants associated with exposure factors such as instrumental variables (IVs) to imply the causal inference between exposure and disease outcomes (19). MR analyses for inferring the causal relationship of GM on multiple diseases have been widely applied due to various findings from large-scale genome-wide association studies (GWASs) to data conducted on GM (20, 21). Previous studies have indicated that some GM are causally associated with T1D (22) and T2D (23). However, there was no evidence that demonstrated whether GM became a potential causal factor on diabetic microvascular complications. Therefore, MR analysis allows us to assess the contribution of GM on diabetic microvascular complications in the present study. Furthermore, this study could facilitate drug discovery and obtain reliable surrogate biomarkers to predict the onset and progression of diabetic microvascular complications, including DKD, DR, and DNP.

Materials and methods

Data sources

Genetic variants for GM were obtained from the MiBioGen consortium, which performed the largest multi-ancestry genome-wide meta-analysis published to date (20). The study included 18,340 individuals, of whom over 70% were of European ancestry in 24 cohorts, targeting three distinct variable regions of the 16S rRNA gene to profile the microbial composition and utilizing direct taxonomic binning for conducting taxonomic classification. In addition, every sample was rarefied to 10,000 reads in all datasets on the interpretation of different sequencing depths. Microbiome quantitative trait loci mapping analysis, including 211 taxa (five levels, in the order of genus, family, order, class, and phylum), was conducted to identify the effect of host genetics on the relative abundance levels of microbial taxa. More details related to the GM data could be found elsewhere (20). All the GWAS summary statistics of diabetic complications in this study were acquired from the FinnGen research project (https://r9.finngen.fi/). Lastly, a total of 4,111 cases and 308,539 controls in DKD, 10,413 cases and 308,633 controls in DR, and 2,843 cases and 271,817 controls in DNP were included.

Ethics statement

The summary-level data involved in this study are free and publicly available for download. The respective institutions have approved the ethics statement of each GWAS in this study. There were no individual-level data in this study; thus, new ethical review board approval was unnecessary.

Instrumental variable selection

The selection of optimal IVs was vital for the robustness and accuracy of the causal association, which conformed to the MR's three principal assumptions (24), specifically for relevance, independence, and exclusion assumption. To explore more relations, a relatively more comprehensive threshold ($p < 1e{-}05$) of single-nucleotide polymorphisms (SNPs) associated with GM was applied (25). Data of the European-based 1,000 Genome Projects were set as the reference panel for performing a linkage disequilibrium (LD) analysis, where SNPs had $r^2 < 0.001$ and the window size was 10,000 kb. *F* statistics not less than 10 represented the notable strength of the selected SNPs for each bacterial taxon, the equation of which is $R^2(N-2)/(1-R^2)$ (26), where R^2 denotes the proportion in exposure variance of each selected IV interpretation and *N* represents the sample size (27). Minor allele frequency (MAF) <0.01 of SNPs was removed, aiming at clearing mutations in less than 1% of the population. Furthermore, palindromic SNPs were removed to prevent distortion of strand orientation or allele coding.

Statistical analysis

Inverse variance weighted (IVW) was the primary method to examine the causal association between GM and diabetic complications based on ratio estimates of each variant (28), which provided a more conservative but robust estimate (29), the *p*-value of which determined the criterion for the existence of a causal association between exposure and outcome. Cochran's *Q* test was used for the assessment of the heterogeneity among IVs, and the random-effects model was applied in the presence of significant heterogeneity; otherwise, the fixed-effects model was used (30). A series of additional MR analyses were conducted for calculating the causal effect values, including the weighted median, MR-Egger regression, simple mode, and weighted mode methods. The weighted median method, the median of the weighted ratio estimates of valid variants as the total weight of the instrument, showed consistent results with IVW in the condition of even up to 50% of invalid IVs (28). The MR-Egger regression test employed a weighted linear regression instead of setting the intercept to zero in IVW and allowed the presence of over 50% of invalid IVs, intercept estimated by MR-Egger regression could serve to estimate the average horizontal effect of pleiotropy (31, 32). The largest cluster of SNPs was applied in simple mode and the weights were assigned to each SNP in weighted mode (33, 34). In scenarios where the beta values for exposure and outcome summary data exhibited significant disparity in distribution, the correct factor will be utilized.

Evaluation of overall horizontal pleiotropy was conducted by the MR pleiotropy residual sum and outlier (MR-PRESSO) global test, and then outlier removal could correct this pleiotropy (25). The intercept from the MR-Egger test further verified the sensitivity; $p_{intercept} < 0.05$ indicated horizontal pleiotropy (31). Directional causal inference judged by the MR Steiger directionality test was made (35). Additionally, the leave-one-out analysis was performed to validate data robustness and avoid affecting significant results via a single SNP (36).

Reverse Mendelian randomized analysis

A reverse MR analysis was also conducted to explore whether the disease outcomes have any causal impact on the GM, especially the identified significant ones. Noteworthily, SNPs related to each genus of diabetic complications at the locus-wide significance threshold ($p < 5e{-}08$) were selected as potential IVs to obtain more comprehensive results (22), which were different from screening the IVs in the pre-MR analysis.

The threshold of statistical significance was identified as $p < 0.05$ and odds ratio (OR) with 95% confidence interval (CI) was regarded as the effect between GM and diabetic complications. False discovery rate (FDR) correction was conducted; a *q*-value of more than 0.1 means no suggestive causal association (37). R software (version 4.3.1) was used for all the above statistical analyses. We used the Strengthening the Reporting of Observational Studies in Epidemiology using Mendelian Randomization (STROBE-MR) checklist published in 2021 as reference (38).

R software (version 4.3.1. R Foundation for Statistical Computing 2023) was utilized in all of the analyses. The packages TwoSampleMR (version 0.5.7), MRPRESSO (version 1.0), vroom (version 1.6.4), grid (version 4.3.1), forestploter (version 1.1.1), data.table (version 1.14.8), phenoscanner (version 1.0), dplyr (version 1.1.2), fdrtool (version 1.2.17), plinkbinr (version 0.0.0.9000), and ieugwasr (version 0.1.5) were used.

Results

Causal effects of GM on diabetic microvascular diseases

A total of 196 bacterial taxa were identified for MR analysis after removing 15 unknown families or genera. All the *F* statistics of the IVs selected were more than 10, indicating no evidence of weak instrumental bias. Meanwhile, over three SNPs of each GM were included for a successful MR-PRESSO test. In addition, the MAFs were all more than 0.01. Lastly, a total of 2,092 IVs were identified, including 9 phyla (114 SNPs), 16 classes (179 SNPs), 20 orders (216 SNPs), 32 families (352 SNPs), and 119 genera (1,231 SNPs). Detailed information of IVs used in the MR analysis for the causal inference is presented in Supplementary Table S1. It was worth noting that the more taxonomically distinct GM was chosen

when GM shared the same SNPs; in other words, we would pick the class *Verrucomicrobiae* instead of the order *Verrucomicrobiales*. An overall view of the MR analysis process and major hypotheses is shown in Figure 1.

Diabetic kidney disease

In the phylum level, only the genetic predicted Bacteroidetes (OR = 1.43, 95% CI = 1.09–1.88, p = 1.08e−02) was causally associated with DKD. As for the class level, we found that the higher genetically predicted Bacteroidia (OR = 1.45, 95% CI = 1.12–1.87, p = 4.57e−03) and Verrucomicrobiae (OR = 1.40, 95% CI = 1.13–1.73, p = 1.80e−03) were identified as higher risks of DKD. Meanwhile, the genetically predicted genera *Catenibacterium* (OR = 1.31, 95% CI = 1.08–1.59, p = 6.40e−03), *Lachnoclostridium* (OR = 1.43, 95% CI = 1.13–1.82, p = 3.11e−03), and *Parasutterella* (OR = 1.27, 95% CI = 1.07–1.51, p = 6.52e−03) were also causally associated with DKD. However, we found that family *Bacteroidaceae* (OR = 0.72, 95% CI = 0.52–0.99, p = 4.64e−02), family *Victivallaceae* (OR = 0.87, 95% CI = 0.77–0.98, p = 2.45e−02), genus *Coprococcus2* (OR = 0.74, 95% CI = 0.58–0.96, p = 2.47e−02), and genus *Lactococcus* (OR = 0.85, 95% CI = 0.73–0.99, p = 3.93e−02) played protective roles in the causal inference between GM and DKD.

Diabetic retinopathy

We found that the genetically predicted class Bacteroidia was causally associated with DR (OR = 1.24, 95% CI = 1.05–1.46, p = 9.75e−03). In the family level, the higher genetically predicted *BacteroidalesS24* (OR = 1.16, 95% CI = 1.02–1.33, p = 2.45e−02), *ClostridialesvadinBB60group* (OR = 1.18, 95% CI = 1.06–1.32, p = 2.62e−03), and *Peptostreptococcaceae* (OR = 1.17, 95% CI = 1.03–1.33, p = 1.90e−02) were related to the higher abundance of DR. In addition, IVW results demonstrated a harmful effect of the host-genetic-driven increase in the genera *Eubacterium nodatum group* (OR = 1.08, 95% CI = 1.01–1.17, p = 3.53e−02), *Actinomyces* (OR = 1.15, 95% CI = 1.01–1.32, p = 3.25e−02), *Olsenella* (OR = 1.10, 95% CI = 1.01–1.20, p = 2.16e−02), *Parasutterella* (OR = 1.12, 95% CI = 1.01–1.25, p = 3.87e−02), *RuminococcaceaeUCG003* (OR = 1.15, 95% CI = 1.00–1.32, p = 4.54e−02), and *RuminococcaceaeUCG011* (OR = 1.15, 95% CI = 1.04–1.28, p = 5.83e−03) on the risk of DKD, except for the genus *Eisenbergiella* (OR = 0.90, 95% CI = 0.82–0.99, p = 3.17e−02) acting as a protective factor.

Diabetic neuropathy

The genetic liability for the family *Acidaminococcaceae* (OR = 0.62, 95% CI = 0.46–0.84, p = 1.76e−03), family *Peptococcaceae* (OR = 0.70, 95% CI = 0.54–0.90, p = 5.65e−03), and genus *Eubacterium coprostanoligenes group* (OR = 0.68, 95% CI = 0.50-0.93, p = 1.61e−02) contributed to a decreased abundance of DNP in the results of IVW analyses. Nevertheless, the higher genetically predicted genera *Alistipes* (OR = 1.65, 95% CI = 1.18–2.31, p = 3.21e−03), *ChristensenellaceaeR_7group* (OR = 1.52, 95% CI = 1.03–2.23, p = 3.28e−02), *Eggerthella* (OR = 1.28, 95% CI = 1.05–1.55, p = 1.42e−02), and *RuminococcaceaeUCG013* (OR = 1.35, 95% CI = 1.01–1.82, p = 4.57e−02) were causally associated with a higher abundance of DNP.

The significant results of the IVW analysis for the causal inference of GM on diabetic microvascular complications are

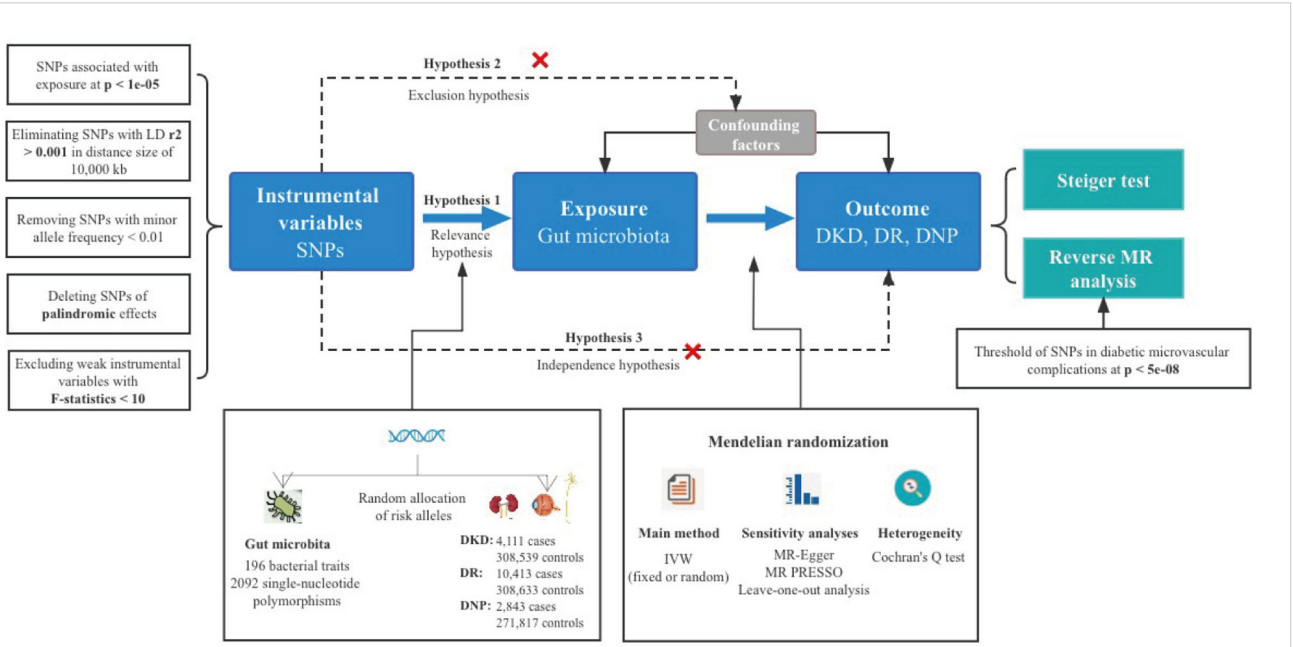

FIGURE 1
Overview of MR analysis process and major hypotheses.

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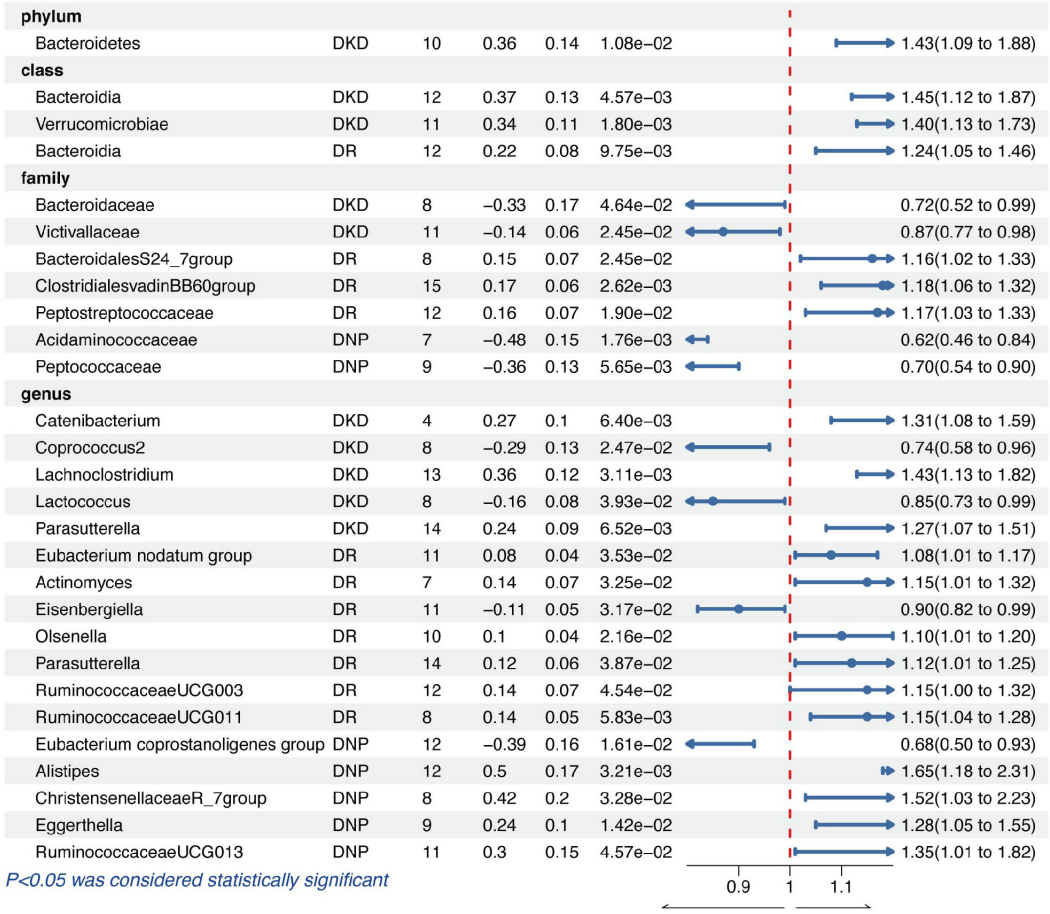

FIGURE 2
The results of the IVW method for causal inference of GM on diabetic microvascular complications.

presented in Figure 2, where fixed-effect models were applied. No significant q-value of the GM was discovered in FDR correction analysis. Results of all MR analyses (p-value of IVW method < 0.05) along with FDR correction for the IVW method are shown in Supplementary Tables S2–S4 and visual inspection of MR analyses is shown in Supplementary Figures S1–S3.

Sensitivity analysis

No evidence of heterogeneity ($p > 0.05$) was observed in Cochran's Q test, thus resulting in the fixed-effect models being used for the causal inference in the IVW analyses. Moreover, no significant horizontal pleiotropy existed in the MR-PRESSO analysis (global test $p > 0.05$), and MR-Egger regression intercept analysis was further verified ($p > 0.05$). Therefore, we could conclude that the results of IVW were authentic in the absence of pleiotropy and heterogeneity. At the same time, the Steiger test indicated no directional causal estimations between GM and diabetic microvascular complications. Summary data of heterogeneity, pleiotropy, and direction analyses are presented in Table 1. The leave-one-out analysis indicated that no single SNP affects the causal inference of GM on diabetic microvascular complications (Supplementary Figures S4–S6).

Reverse causal effects of diabetic microvascular diseases on GM

When diabetic microvascular complications were set as exposure and GM as outcome, a total of 6 IVs on DKD, 17 IVs on DR, and 4 IVs on DNP were included according to the strict quality selection (Supplementary Table S5).

Figure 3 shows that genetically predicted diabetic microvascular complications were causally associated with some other GM in the IVW results. Summary data of MR analysis between DKD, DR, DNP, and GM are separately presented in Supplementary Tables S6 and S7. DKD was a protective factor for *Eubacterium ventriosum group* (OR = 0.96, 95% CI = 0.92–1.00, p = 3.73e−02) and a risk factor for *Anaerofilum* (OR = 1.09, 95% CI = 1.03–1.16, p = 4.84e−03) in the genus level.

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TABLE 1 Heterogeneity, pleiotropy and directional analyses of GM on diabetic microvascular complications.

Level	Exposure	Outcome	Heterogeneity P for Cochran's Q	MRegger_ intercept	Horizontal pleiotrophy P for Egger intercept	steiger_pval	MRPRESSO
phylum	*Bacteroidetes*	DKD	0.848	-0.003	0.907	3.33E-65	0.865
class	*Bacteroidia*	DKD	0.906	-0.002	0.914	3.26E-73	0.92
class	*Verrucomicrobiae*	DKD	0.451	0.034	0.263	1.16E-51	0.481
family	*Bacteroidaceae*	DKD	0.284	-0.021	0.738	3.60E-34	0.338
family	*Victivallaceae*	DKD	0.710	0.000	0.996	5.98E-56	0.718
genus	*Catenibacterium*	DKD	0.902	-0.103	0.586	3.73E-19	0.911
genus	*Coprococcus2*	DKD	0.701	0.042	0.590	7.31E-34	0.745
genus	*Lachnoclostridium*	DKD	0.707	0.005	0.863	1.87E-52	0.733
genus	*Lactococcus*	DKD	0.782	0.046	0.389	5.40E-39	0.823
genus	*Parasutterella*	DKD	0.952	0.018	0.385	1.02E-68	0.941
class	*Bacteroidia*	DR	0.865	-0.013	0.338	1.68E-73	0.865
family	*BacteroidalesS24_7group*	DR	0.359	0.009	0.758	1.61E-39	0.415
family	*ClostridialesvadinBB60group*	DR	0.720	0.017	0.238	1.51E-72	0.749
family	*Peptostreptococcaceae*	DR	0.780	0.027	0.051	1.10E-72	0.761
genus	*Eubacterium nodatum group*	DR	0.889	-0.023	0.381	3.48E-53	0.896
genus	*Actinomyces*	DR	0.791	-0.015	0.442	1.09E-33	0.821
genus	*Eisenbergiella*	DR	0.425	-0.008	0.852	2.09E-49	0.466
genus	*Olsenella*	DR	0.330	0.028	0.156	3.34E-47	0.385
genus	*Parasutterella*	DR	0.963	0.004	0.787	1.61E-69	0.96
genus	*RuminococcaceaeUCG003*	DR	0.629	0.013	0.476	1.55E-58	0.631
genus	*RuminococcaceaeUCG011*	DR	0.208	0.029	0.441	2.54E-39	0.249
family	*Acidaminococcaceae*	DNP	0.551	-0.078	0.148	1.11E-30	0.595
family	*Peptococcaceae*	DNP	0.804	-0.003	0.940	6.43E-48	0.816
genus	*Eubacterium coprostanoligenes group*	DNP	0.676	-0.008	0.828	1.72E-50	0.68
genus	*Alistipes*	DNP	0.663	0.039	0.444	5.65E-46	0.691
genus	*ChristensenellaceaeR_7group*	DNP	0.426	-0.019	0.746	5.20E-32	0.477
genus	*Eggerthella*	DNP	0.504	-0.032	0.536	1.50E-40	0.502
genus	*RuminococcaceaeUCG013*	DNP	0.847	-0.022	0.534	2.80E-53	0.854

The IVW results indicated that a higher genetically predicted DR, on the one hand, had a beneficial role on the phylum *Verrucomicrobia* (OR = 0.94, 95% CI = 0.89–0.98, p = 6.73e–03), the class *Verrucomicrobiae* (OR = 0.93, 95% CI = 0.88–0.97, p = 3.14e–03), the families *Family XIII* (OR = 0.95, 95% CI = 0.91–0.99, p = 1.54e–02) and *Verrucomicrobiaceae* (OR = 0.93, 95% CI = 0.89-0.98, p = 3.19e–03), and the genera *Enterorhabdus* (OR = 0.93, 95% CI = 0.88–0.99, p = 2.77e–02), *Eisenbergiella* (OR = 0.92, 95% CI = 0.85–0.99, p = 2.01e–02), *Eubacterium hallii group* (OR = 0.95, 95% CI = 0.91–0.99, p = 2.96e–02), and *Akkermansia* (OR = 0.93, 95% CI = 0.88–0.97, p = 3.02e–03); on the other hand, DR had an adverse effect on the family *Clostridiaceae1* (OR =1.08, 95% CI = 1.03-1.13, p = 1.44e–03) and the genera *Faecalibacterium* (OR =1.04, 95% CI = 1.00–1.09, p = 2.88e–02), *Anaerofilum* (OR =1.10, 95% CI = 1.02–1.19, p = 9.35e–03), *Catenibacterium* (OR =1.10, 95% CI = 1.00–1.22, p = 4.98e–02), *Veillonella* (OR =1.07, 95% CI = 1.00–1.13, p = 4.07e–02), and *Clostridium sensustricto1* (OR =1.08, 95% CI = 1.03–1.13, p = 2.36e–03).

In the order level, DNP was causally associated with NB1n (OR =1.07, 95% CI = 1.00–1.14, p = 3.77e–02). As for the

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exposure	outcome	nsnp	b	se	pval	OR(95%CI)
	phylum					
DR	Verrucomicrobia	16	−0.066	0.024	6.73e−03	0.94(0.89 to 0.98)
	class					
DR	Verrucomicrobiae	16	−0.074	0.025	3.14e−03	0.93(0.88 to 0.97)
	order					
DNP	NB1n	4	0.066	0.032	3.77e−02	1.07(1.00 to 1.14)
	family					
DR	Clostridiaceae1	17	0.076	0.024	1.44e−03	1.08(1.03 to 1.13)
DR	Family XIII	17	−0.051	0.021	1.54e−02	0.95(0.91 to 0.99)
DR	Verrucomicrobiaceae	16	−0.074	0.025	3.19e−03	0.93(0.89 to 0.98)
	genus					
DKD	Eubacterium ventriosum group	6	−0.042	0.02	3.73e−02	0.96(0.92 to 1.00)
DKD	Anaerofilum	6	0.09	0.032	4.84e−03	1.09(1.03 to 1.16)
DR	Faecalibacterium	17	0.044	0.02	2.88e−02	1.04(1.00 to 1.09)
DR	Anaerofilum	16	0.099	0.038	9.35e−03	1.10(1.02 to 1.19)
DR	Enterorhabdus	16	−0.069	0.031	2.77e−02	0.93(0.88 to 0.99)
DR	Catenibacterium	14	0.099	0.05	4.98e−02	1.10(1.00 to 1.22)
DR	Eisenbergiella	16	−0.085	0.036	2.01e−02	0.92(0.85 to 0.99)
DR	Eubacterium hallii group	17	−0.052	0.024	2.96e−02	0.95(0.91 to 0.99)
DR	Akkermansia	16	−0.074	0.025	3.02e−03	0.93(0.88 to 0.97)
DR	Veillonella	16	0.064	0.031	4.07e−02	1.07(1.00 to 1.13)
DR	Clostridium sensustricto1	17	0.076	0.025	2.36e−03	1.08(1.03 to 1.13)
DNP	Ruminococcus2	4	0.056	0.025	2.71e−02	1.06(1.01 to 1.11)
DNP	Subdoligranulum	82	0.017	0.009	4.68e−02	1.02(1.00 to 1.03)
DNP	Eubacterium coprostanoligenes group	82	0.021	0.008	1.33e−02	1.02(1.00 to 1.04)
DNP	Actinomyces	4	0.055	0.028	4.65e−02	1.06(1.00 to 1.12)
DNP	Streptococcus	4	0.054	0.027	4.77e−02	1.05(1.00 to 1.11)
DNP	Eubacterium nodatum group	3	0.095	0.043	2.77e−02	1.10(1.01 to 1.20)

P<0.05 was considered statistically significant

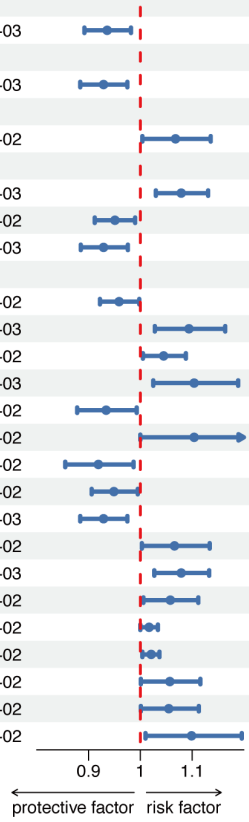

FIGURE 3
The results of the IVW method given as odds ratio (OR) and 95% confidence interval (CI) for causal inference of diabetic microvascular complications on GM. DR, diabetic retinopathy; DNP, diabetic neuropathy; DKD, diabetic kidney disease.

biological genus classifications, the IVW results indicated that DNP was causally associated with *Ruminococcus2* (OR =1.06, 95% CI = 1.01–1.11, p = 2.71e−02), *Subdoligranulum* (OR =1.02, 95% CI = 1.00–1.03, p = 4.68e−02), *Eubacterium coprostanoligenes group* (OR =1.02, 95% CI = 1.00–1.04, p = 1.33e−02), *Actinomyces* (OR =1.06, 95% CI = 1.00–1.12, p = 4.65e−02), *Streptococcus* (OR =1.05, 95% CI = 1.00–1.11, p = 4.77e−02), and *Eubacterium nodatum group* (OR = 1.10, 95% CI = 1.01–1.20, p = 2.77e−02).

Cochrane's Q test, MR-PRESSO, and MR-Egger regression intercept analysis further demonstrated that no evidence of heterogeneity and pleiotropy existed. Meanwhile, the Steiger test showed no directional causal estimations. Summary data of heterogeneity, pleiotropy, and direction analyses are presented in Supplementary Table S8.

Discussion

To the best of our knowledge, this is the first MR study to evaluate the causal inferences between diabetic microvascular complications and GM from a genetic perspective using the summary statistics of diseases from the FinnGen consortium R9 release data and GM from the largest GWAS meta-analysis conducted by the MiBioGen consortium. Our research mainly discussed the diabetic microvascular complications and the published article discussed six complications (including acute complications of diabetes). Although we chose the same GWAS summary data, we have more cases and controls with DR compared with those in the article of PMID 38481313 (39). Moreover, we conducted the Steiger test and reverse MR analysis to explore the genetically predicted GM on the diabetic microvascular complications.

This two-sample MR analysis provided reasonable evidence that 28 genetically predicted abundance of specific GM play non-negligible roles in the occurrence of DKD, DR, and DNP. Similarly, reverse MR analysis indicated that genetic liability to three chronic microvascular complications was causally associated with 23 GM. In particular, the IVW method indicated a bi-directional causal relationship between *Eisenbergiella* and DR. Amazingly, *Eubacterium coprostanoligenes group* and DNP presented contradictory effects on the causal inference via IVW analysis. These results could bring about implications for new effective treatments to counter DM-associated chronic microvascular complications. Visual results of the causality inference between GM and diabetic microvascular complications are shown in Figure 4.

DKD, a devastating complication of T1D and T2D and a leading cause of end-stage renal disease, occurs in 20% to 50% of patients with DM (40). Few treatment options were available to better stop or delay the onset and progression of DKD. In our study, the family

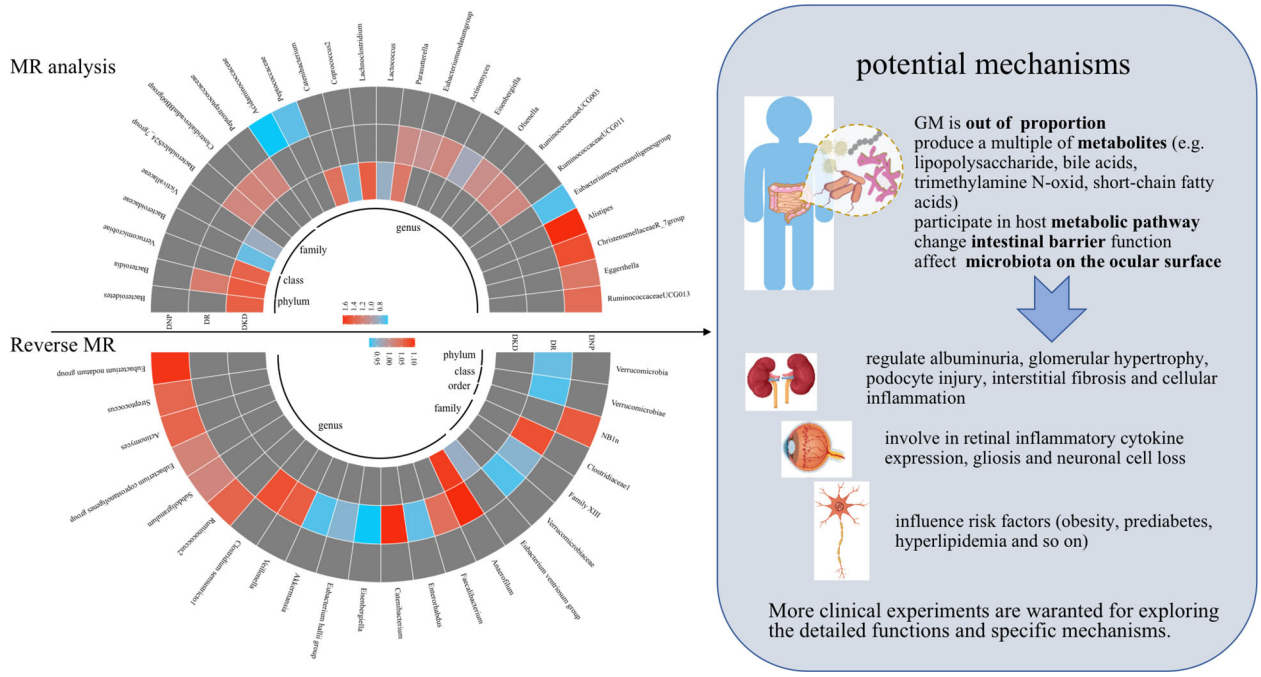

FIGURE 4
Visual results of the causality inference between GM and diabetic microvascular complications were shown on the left and potential mechanisms were presented on the right. Red represents risk factors, blue represents protective factors, and gray represents negative causal inference between disease and GM. MR, Mendelian randomization.

Bacteroidaceae showed an opposite causal inference from its phylum and class levels. Although *Catenibacterium*, *Lachnoclostridium*, *Coprococcus2*, and *Lactococcus* belonged to Firmicutes, they presented different causal relationships on DKD. Previous studies (41, 42) indicated that the relative abundance of Bacteroidetes decreased and that of Firmicutes increased in DKD compared with healthy individuals and in DM without kidney diseases, which was partly consistent with our results. Abnormal ratios of Bacteroidetes/Firmicutes were decreased in rats with chronic kidney disease compared to controls, which was related to increased acetate- and butyrate-producing bacteria (43). This discrepancy could be attributed to different species, complicated diet habits, and the living environment of people. Our results also found that Verrucomicrobiae and *Parasutterella* possibly played harmful roles on DKD, whereas *Victivallaceae* had suggestive protective effects on DKD. A translational human study (44) indicated that *Parasutterella* abundance was positively associated with obesity and T2D, where fatty acid biosynthesis pathway and L-cysteine might be relevant. Similar to the analysis of GM on patients with DKD, the abundance of Verrucomicrobia and Proteobacteria was relatively increased in patients with DKD in comparison with healthy individuals (45).

Furthermore, a multitude of metabolites produced by GM are essential mediators in the crosstalk between the microbial and host environment, such as lipopolysaccharide, bile acids, trimethylamine N-oxide, and short-chain fatty acids, regulating albuminuria, glomerular hypertrophy, podocyte injury, interstitial fibrosis, and cellular inflammation (46–48).

Bacteroides participated in the synthesis of fatty acids, such as acetate, propionate, and butyrate (49, 50). Olfr-78 receptors did not respond to butyrate but were more sensitive to propionate and acetate. Acetate was in the dysregulation of the dynamic homeostasis of fatty acid metabolism by activating Olfr-78 receptors, which led to tubulointerstitial injury in DKD (51). This might be a potential mechanism by which bacillus-like organisms affect diabetic nephropathy.

DR is the dominant cause of preventable blindness in adults and identified in a third of people with DM (52). Five years after the diagnosis of DM, the number of children and adolescents who progressed to DR increased rapidly (53); thus, annual screening with fundus photography and regulation of GM from diet or lifestyle are especially necessary. Substantial microorganisms are enriched not only on the intestinal but also on the ocular surface. The ocular flora plays an important role in the regulation of ocular immunity and prevention of pathogens, especially in the conjunctiva and cornea (54). Studies have shown that patients with diabetes with diabetic complications have higher conjunctival flora than patients with type 2 diabetes without complications (55). Moreover, studies discovered that the primary composition of microbiota on the ocular surface is Proteobacteria and Actinobacteria (56), accounting for over 87% of all microorganisms that exist in the eye along with Firmicutes (57). Modulation of microbiota through oral feeding of *Lactobacillus paracasei* secreting Ang-(1-7) could reduce retinal inflammatory cytokine expression and retinal gliosis and block neuronal cell loss (58).

Our results concluded that Bacteroidetes (Bacteroidia and *BacteroidalesS24*), Firmicutes (including *ClostridialesvadinBB60group*, *Peptostreptococcaceae*, and *Eubacterium nodatum group*), Actinobacteria (including *Actinomyces* and *Olsenella*), *Parasutterella*, and Verrucomicrobia (including *RuminococcaceaeUCG011* and

RuminococcaceaeUCG003) were the potentially detrimental bacteria, except for *Eisenbergiella*, acting as the latent protective bacteria on the occurrence and progression of DR via causal inference. Beli et al. discovered a significant increase of Bacteroidetes and Verrucomicrobia on DR with the decrease of acellular capillaries and leukocyte infiltration (59), which is similar to our results. Moreover, Firmicutes was a risk microbiota in the development of DR. The difference from our results was possibly attributed to the mouse models used, being kept in a sterile environment, and fixed diets. Different from a previously reported MR analysis (60), the results of our study were more comprehensive because we chose the updated data in the FinnGen research project and we all agreed that *RuminococcaceaeUCG011* was a risk factor for the occurrence and progression of DR. Additionally, IVs are enough for us to conduct reverse MR analyses in our study. Interestingly, we discovered that *Parasutterella* played a harmful role on the progression of DKD and DR considering the possible reasons of change in fatty acid biosynthesis and L-cysteine in patients with DM (44). However, whether there is a specific reason for their progression other than DNP remains to be further explored.

DNP, which damages the diffuse and focal nervous system, is the most common complication occurring in up to 50% of individuals with DM, and distal symmetric polyneuropathy is the main characteristic (61). It can also affect other organs, resulting in cardiac autonomic neuropathy accompanied by weakness and orthostatic tachycardia, gastrointestinal autonomic dysfunction including early satiety with poor appetite and nausea, esophageal dysfunction with difficulty swallowing, bladder dysfunction, and sudomotor autonomic disturbance, among others (62). The current treatment of DNP is limited, and corresponding studies on GM that explore the ideal intervention targets and preventive strategies are on the rise. Our results indicated that the lower abundance of *Acidaminococcaceae*, *Peptococcaceae*, and *Eubacterium coprostanoligenes group*, and the higher abundance of *Alistipes*, *ChristensenellaceaeR_7group*, *Eggerthella*, and *RuminococcaceaeUCG013* were causally associated with appearance and deterioration of DNP. Ma et al. discovered no significance of high blood glucose and painful hypersensitivity in animals induced by streptozotocin as major depletion of GM in comparison with controls (63). However, as far as we know, there are no relevant studies observed between our chosen GM and DNP, but they are associated with relative risk factors. The relationship between gut dysbiosis, neuronal damage, and dyskinesia is not yet fully understood, but GM clearly plays an important role in maintaining the function of the enteric nervous system (64). In a recent study, Nyavor et al. (65) found a reduction in inhibitory neuromuscular transmission and a loss of inhibitory motor neurons in muscles in rats fed a high-fat diet. High-fat diets also lead to microbiological imbalances in the bacterial flora, such as increased numbers of *Aspergillus*, *Lactobacillus*, and *Bifidobacterium*. These changes are associated with neuropathy and intestinal motility disorders.

Insulin resistance is closely linked to the onset and progression of DNP (66, 67). Yuan et al. indicated that Firmicutes was decreased and Bacteroidetes was increased in insulin-resistant subjects compared with insulin-sensitive individuals (68). *Acidaminococcaceae* and *Peptococcaceae* belonged to Firmicutes, whereas *Alistipes* was part of Bacteroidetes. Furthermore, *Peptococcaceae* was significantly more prevalent and *Alistipes* was less prevalent in individuals with insulin resistance via influencing serum concentrations of angiopoietin-like 4 and adropin, which was consistent with our causal inference of GM on DNP. *ChristensenellaceaeR_7group* was positively correlated with obesity and would be a potential therapeutic target of traditional Chinese medicine to relieve obesity (69). Although no research observed the relationship between *RuminococcaceaeUCG013* and DNP, a study demonstrated that abnormalities of *Ruminococcaceae* caused cognitive dysfunction in DNP rats (70). In general, the causality of GM found in our study needs to be further verified.

The above positive strains showed no causal inference in reverse MR analyses except for *Eisenbergiella* and *Eubacterium coprostanoligenes group*. *Eisenbergiella* showed a bi-directional causal relationship in the reverse MR analysis. A plethora of studies revealed that alterations of the abundance of *Eisenbergiella* led to numerous diseases, including multiple sclerosis, rheumatoid arthritis, and autism (71–73). Upregulated *Eisenbergiella* affected fatty acid metabolism by the production of short-chain fatty acids to reduce obesity, an important risk factor for DM. Therefore, we can conclude that it could be a non-invasive biomarker or a potential target for the treatment of patients with DR. Our results implied an opposite mutual causal relationship between *Eubacterium coprostanoligenes group* and DNP.

Although no studies of *Eubacterium coprostanoligenes group* were relevant to neuropathy, some studies indicated improvement of dyslipidemia induced by high-fat diet (74) and amelioration of fasting blood glucose, hemoglobin, serum levels of endotoxin, interlukin-6, tumor necrosis factor-α, and interlukin-1β in prediabetes (75). Hyperlipidemia and prediabetes are risk factors for DNP (66, 76). As a result, we recognize that *Eubacterium coprostanoligenes group* could possibly mediate the beneficial effects of DNP. MR analyses for diabetic microvascular complications on GM substantiated positive effects on the other GM. However, OR generally ranged from 0.9 to 1.1, demonstrating little causal relationship. These strains were still novel diagnostic biomarkers of diseases and therapeutic breakthrough.

Inevitably, it should be noted that our study has some limitations that could have affected the results. Firstly, the GWAS included only individuals of European descent; thus, the generation of our findings to other races is limited. Secondly, because the summary statistics instead of raw data were utilized in the analysis, microvascular complications caused by T1DM or T2DM were lack of specific information in the FinnGen database, which restricted the further subgroup analyses. Thirdly, a relatively lenient GWAS significance threshold ($p < 1e{-}05$) was set for more genetic variations in IVs in order to perform horizontal pleiotropy detection and sensitivity analysis. Thus, FDR correction was utilized to lower the probability of being false positive. Fourthly, GM together with its metabolites and by-products played important roles on the onset and progression of diabetic microvascular complications. However, our study only explored the causal relationship between microvascular complications and GM. Therefore, it would be helpful to perform causal association between GM and diabetic microvascular complications in diverse European and non-European populations for more generalizability. Multivariable MR analysis can also be considered for the corresponding metabolites in

future endeavors. Lastly, although amplicon sequence variant-level analysis provided better resolution and more accurate results in the 16S rRNA gene-based microbiota studies in comparison with taxa-level analysis, we only obtained datasets of taxa.

Conclusion

The present study gave credence to the concept that GM may be a promising therapy in diabetic microvascular complications. Further experiments of GM therapy on diabetic microvascular complications are warranted to elucidate their effects and specific mechanisms.

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.

Ethics statement

All the GWAS summary statistics of diabetic complications in this study were acquired from the FinnGen research project (https://r9.finngen.fi/). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin. The summary-level data involved in this study are free of identified and publicly available for download. Respective institutions have approved the ethics statement of Each GWAS in this study. There was no individual-level data in this study, thus, new ethical review board approval was unnecessary.

Author contributions

PZ: Conceptualization, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. ZH: Data curation, Software, Writing – original draft. WX: Methodology, Writing – original draft. YC: Formal analysis, Writing – review & editing. ZZ: Supervision, Visualization, Writing – review & editing. JY: Funding acquisition, Supervision, Validation, Writing – review & editing, Conceptualization.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1364280/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Scatter plots for the causal association between **(A)** *Bacteroidetes*, **(B)** *Bacteroidia*, **(C)** *Verrucomicrobiae*, **(D)** *Bacteroidaceae*, **(E)** *Victivallaceae*, **(F)** *Catenibacterium*, **(G)** *Coprococcus2*, **(H)** *Lachnoclostridium*, **(I)** *Lactococcus*, and **(J)** *Parasutterella* and DKD. The slope of each line corresponds to the estimated MR effect in different models.

SUPPLEMENTARY FIGURE 2
Scatter plots for the causal association between **(A)** *Bacteroidia*, **(B)** *BacteroidalesS24_7group*, **(C)** *ClostridialesvadinBB60group*, **(D)** *Peptostreptococcaceae*, **(E)** *Eubacterium nodatum group*, **(F)** *Actinomyces*, **(G)** *Eisenbergiella*, **(H)** *Olsenella*, **(I)** *Parasutterella*, **(J)** *RuminococcaceaeUCG003*, and **(K)** *RuminococcaceaeUCG011* and DR. The slope of each line corresponds to the estimated MR effect in different models.

SUPPLEMENTARY FIGURE 3
Scatter plots for the causal association between **(A)** *Acidaminococcaceae*, **(B)** Peptococcaceae, **(C)** *Eubacterium coprostanoligenes group*, **(D)** *Alistipes*, **(E)** *ChristensenellaceaeR_7group*, **(F)** *Eggerthella*, and **(G)** *RuminococcaceaeUCG013* and DNP. The slope of each line corresponds to the estimated MR effect in different models.

SUPPLEMENTARY FIGURE 4
MR leave-one-out sensitivity analysis for **(A)** *Bacteroidetes*, **(B)** *Bacteroidia*, **(C)** *Verrucomicrobiae*, **(D)** *Bacteroidaceae*, **(E)** *Victivallaceae*, **(F)** *Catenibacterium*, **(G)** Coprococcus2, **(H)** *Lachnoclostridium*, **(I)** *Lactococcus*, and **(J)** *Parasutterella* on DKD. Calculate the MR results of the remaining IVs after removing the IVs one by one.

SUPPLEMENTARY FIGURE 5
MR leave-one-out sensitivity analysis for **(A)** *Bacteroidia*, **(B)** *BacteroidalesS24_7group*, **(C)** *ClostridialesvadinBB60group*, **(D)** Peptostreptococcaceae, **(E)** *Eubacterium nodatum group*, **(F)** Actinomyces, **(G)** Eisenbergiella, **(H)** Olsenella, **(I)** *Parasutterella*, **(J)** *RuminococcaceaeUCG003*, and **(K)** *RuminococcaceaeUCG011* on DR. Calculate the MR results of the remaining IVs after removing the IVs one by one.

SUPPLEMENTARY FIGURE 6
MR leave-one-out sensitivity analysis for **(A)** *Acidaminococcaceae*, **(B)** Peptococcaceae, **(C)** *Eubacterium coprostanoligenes group*, **(D)** *Alistipes*, **(E)** *ChristensenellaceaeR_7group*, **(F)** *Eggerthella*, and **(G)** *RuminococcaceaeUCG013* on DNP. Calculate the MR results of the remaining IVs after removing the IVs one by one.

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Effect of oral metformin on gut microbiota characteristics and metabolite fractions in normal-weight type 2 diabetic mellitus patients

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Background and aims: To analyze the effect of oral metformin on changes in gut microbiota characteristics and metabolite composition in normal weight type 2 diabetic patients.

Methods: T2DM patients in the cross-sectional study were given metformin for 12 weeks. Patients with unmedicated T2DM were used as a control group to observe the metrics of T2DM patients treated with metformin regimen. 16S rDNA high-throughput gene sequencing of fecal gut microbiota of the study subjects was performed by llumina NovaSeq6000 platform. Targeted macro-metabolomics was performed on 14 cases of each of the gut microbiota metabolites of the study subjects using UPLC-MS/MS technology. Correlations between the characteristics of the gut microbiota and its metabolites, basic human parameters, glycolipid metabolism indicators, and inflammatory factors were analyzed using spearman analysis.

Results: Glycolipid metabolism indexes and inflammatory factors were higher in normal-weight T2DM patients than in the healthy population ($P<0.05$), but body weight, BMI, waist circumference, and inflammatory factor concentrations were lower in normal-weight T2DM patients than in obese T2DM patients ($P<0.05$). Treatment with metformin in T2DM patients improved glycolipid metabolism, but the recovery of glycolipid metabolism was more pronounced in obese T2DM patients. None of the differences in α-diversity indexes were statistically significant ($P>0.05$), and the differences in β-diversity were statistically significant ($P<0.05$). Community diversity and species richness recovered after metformin intervention compared to before, and were closer to the healthy population. We found that *Anaerostipes*/Xylose/Ribulose/Xylulose may play an important role in the treatment of normal-weight T2DM with metformin by improving glycemic lipids and reducing inflammation. And Metformin may play a role in obese T2DM through *Romboutsia*, medium-chain fatty acids (octanoic acid, decanoic acid, and dodecanoic acid).

Conclusion: Gut microbial dysbiosis and metabolic disorders were closely related to glucose-lipid metabolism and systemic inflammatory response in normal-weight T2DM patients. Metformin treatment improved glucose metabolism levels, systemic inflammation levels in T2DM patients, closer to the state of healthy population. This effect may be mediated by influencing the gut microbiota and microbial host co-metabolites, mainly associated with *Anaerostipes* and xylose/Ribulose/Xylulose. Metformin may exert its effects through different pathways in normal-weight versus obese T2DM patients.

KEYWORDS

type 2 diabetes mellitus, metformin, metabolomics, gut microbiota, body mass index

Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by prolonged hyperglycemia due to disorders of glucose metabolism caused by insulin deficiency and/or impaired insulin bioactivity, often accompanied by a variety of metabolic disorders, which seriously jeopardizes the life and health of patients. Some studies (1) have shown that the increase in the number of type 2 diabetes patients worldwide is related to factors such as overweight and obesity, and its mechanism may be its own insulin resistance. However, there is growing evidence that T2DM can also occur in people with normal body mass index (BMI) that and that normal-weight patients with T2DM exhibit a higher propensity for pancreatic β-cell failure, as well as having lower waist circumference, waist-to-hip ratio, total and visceral fat levels, and fasting insulin, fasting C-peptide, and insulin resistance indices (HOMA2-IR) compared with obese T2DM (2, 3). Since normal-weight T2DM patients do not require weight-loss treatment and their metabolic disorders are less severe than those of obese T2DM patients, they require different treatments compared with obese T2DM patients. Currently, most of the studies at home and abroad have been conducted on obese or overweight T2DM patients, and there is a lack of literature support for studies on normal-weight T2DM patients; therefore, finding more effective hypoglycemic agents in glucose-lowering regimens for normal-weight T2DM patients has become a hot topic in current research.

It is well known that gut microbiota plays an important role in energy acquisition from food and immunomodulation. As an invisible organ in the human body, the dysregulation of gut microbiota is involved in the development of various metabolic diseases in the human body, such as obesity, insulin resistance, T2DM, inflammatory bowel disease, and non-alcoholic fatty liver disease, etc. The short-chain fatty acids (SCFA) and amino acid metabolism derivatives produced by gut microorganisms can also act as messengers to regulate the body's glucose metabolism and affect the development of T2DM. Some studies have found that in healthy populations, the gut microbiota of normal-weight and obese populations had significant differences, with the abundance of the thick-walled phylum in normal-weight populations being lower than that in obese populations, and the abundance of the phylum Bacteroidetes being higher (4). The results showed that the composition of the gut microbiota and its metabolites were significantly different between diabetic obese mice and normal-weight mice (5). While the gut microbiota metabolites of short-chain fatty acids, fecal bile acid concentrations were significantly lower, and phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and palmitocarnitine were elevated in patients with T2DM compared with healthy subjects (6). Therefore, we venture to predict that there may be a difference in gut microbiota between normal-weight and obese patients in the T2DM population, but there is a lack of literature support regarding normal-weight T2DM patients in population-based studies to be further explored.

Metformin is the most widely used hypoglycemic agent for the treatment of patients with T2DM, and is recommended as the first-line therapeutic agent because of its obvious hypoglycemic effect, relative safety, and low cost. The antidiabetic mechanism of action of metformin may be through the activation of adenosine monophosphate-activated protein kinase (AMPK), inhibition of hepatic glucose gluconeogenesis, increase insulin sensitivity, and enhancement of peripheral glucose uptake in the liver and skeletal muscle. Currently, it had been shown that the efficacy of metformin was not related to body weight, but Deng et al. found different results in their study, showing that BMI was negatively correlated with the magnitude of decrease in glycosylated hemoglobin and fasting glucose after metformin intervention (7, 8). Recent studies showed that metformin significantly improved the gut microbiota of T2DM patients, which may promote the improvement of immune status through the modulation of gut microbiota, which in turn exerts a glucose-lowering mechanism, but this interaction is still unclear (9). Thomas (10) and Ilze (11) et al. showed that the composition of the gut microbiota of healthy young men changed significantly with metformin, such as a decrease in the abundance of

Enterobacteriaceae and an increase in the abundance of Prevotella and Bifidobacteria, but returned to baseline levels after discontinuation of the treatment, suggesting that metformin had a significant effect on the gut microbiota of healthy populations, and that the effect disappeared after discontinuation of the drug. Similarly, in a double-blind trial, we observed a decrease in α diversity and an increase in Ehrlichia, Bifidobacterium, and Akkermannia and a decrease in Enterobacteriaceae in obese T2DM patients treated with metformin (11). However, what is the effect of metformin on the gut microbiota of normal-weight T2DM patients, and whether it can improve metabolic indexes such as blood glucose in normal-weight T2DM patients by regulating the gut microbiota needs to be further explored. In addition, it had also been found that Metformin promoted changes in gut microbial metabolites in T2DM patients, and the results of macrogenomics and metabolomics analyses revealed that lipopolysaccharide biosynthesis was increased and significantly increased butyrate and propionate in the gut of T2DM patients (11). Therefore, we can propose a hypothesis that the application of metformin therapy in normal-weight T2DM patients may be through the pathway of gut microbiota and its metabolites, but not the same as in obese T2DM patients.

In summary, our study intends to observe the characteristics of gut microbiota in normal-weight T2DM patients, and to explore the effects of gut microbiota and its metabolites on the body's glucose-lipid metabolism after metformin intervention, so as to explore the pathogenesis of the disease and the antidiabetic effect of metformin, and to explore the correlation between them. It will provide a basis for further revealing the relationship between diabetes and gut microbiota and guiding the precise treatment program for normal-weight T2DM patients.

Materials and methods

Participants

This study was a cross-sectional plus prospective study. Cross-sectional study:60 T2DM patients with a disease duration of ≤5 years admitted to the outpatient clinic or inpatient department of the Department of Endocrinology of Changzhi Medical College Affiliated Heji Hospital from December 2018 to June 2023 (30 cases in the obese group with a BMI of ≥28 kg/m^2 and 30 cases in the normal group with a BMI of 18.5 kg/m^2 ≤ 24 kg/m^2) were selected as the study subjects, and a healthy population was selected from those who had a medical examination at the medical checkup center of the hospital during the same period as a normal control persons (NCP). Healthy people were selected as the normal control persons (NCP).Written informed consent was obtained from all participants prior to enrollment. The study was performed in adherence to the principles of the Declaration of Helsinki with regard to ethical research involving human subjects, and study protocols were approved by the Medical Ethics Committee of Heji Hospital (June 4, 2020, Approval no. 4).

NNT group (n=30): normal-weight T2DM patients;UNT group (n=30): obese T2DM patients;NCP group (n=20): normal control persons. Prospective study: Sixty patients with T2DM from the cross-sectional study were given metformin alone for 12 weeks. NMT group (n=30): metformin monotherapy in normal-weight T2DM patients; MET group (n=30): metformin monotherapy in obese T2DM patients. Medication regimen: Metformin 0.5g/dose 3 times/day with meals, or adjusted according to blood glucose, maximum dose not exceeding 2g/day.

Inclusion criteria for patients with T2DM: 1.Patients with T2DM meeting the diagnostic criteria for diabetes mellitus recommended by the WHO in 1999;2.11% > glycated hemoglobin (HbAlc) ≥6.5%;3. Meet the diagnostic criteria for obesity in China: BMI (kg/m^2) ≥28 and waist circumference ≥85cm for men and ≥80cm for women, and meet the diagnostic criteria for normal body weight in China: 18.5kg/m^2 ≤ BMI <24kg/m^2 and waist circumference <85cm for men and <80cm for women;4. Age between 18-75 years old;5. Disease duration ≤5 years, no previous use of any hypoglycemic drugs;6. No application of antibiotics, microbial live bacterial preparations, etc. in the last 6 months. Inclusion criteria for the NCP population: 1. Normal results of the oral glucose tolerance test, normal people with no diabetes mellitus and no family history of autoimmunity;2. Meets the diagnostic criteria for normal body weight in China: 18.5kg/m^2 ≤BMI <24kg/m^2, and age, gender, and geographic area are matched with T2DM patients.

Exclusion criteria for T2DM patients:1. type 1 diabetes mellitus, diabetic acidosis, patients with diabetic gastroparesis, hidden autoimmune diabetes mellitus in adults, gestational diabetes mellitus and other types of diabetes mellitus etc.;2.Suffering from severe hepatobiliary, gastrointestinal diseases, such as, inflammatory bowel disease, ulcerative colitis, Crohn's, bacillary dysentery, intestinal obstruction, etc., without history of pancreatitis, history of constipation, alcoholism;3. Long-term use of antibiotics and microbial preparations. Patients who used antibiotics (>7 days) or supplemented with live microbial agents (>7 days) within 6 months of sampling were excluded from the study. If the above agents were used for a short period of time (≤7 days), they could be withdrawn from the study for 4 weeks and then re-investigated.;4. Hypersensitivity to GLP-1RA and metformin and its adjuvants;5. Renal insufficiency, blood creatinine level: male >132.61umol/L (1.5mg/dl), female >123.8umol/L (1.4mg/dl), or glomerular filtration rate [GFR<60ml/(min×1.73m^2)], serum ALT or AST exceeds the upper limit of normal more than 3 times, or have severe hepatic insufficiency;6. History of surgery and trauma within the last six months;7. Previous medullary thyroid carcinoma or family history of medullary thyroid carcinoma, multiple endocrine neoplasia syndrome, combined with severe endocrine system diseases such as thyroid, adrenal gland, pituitary gland, etc.; (e.g. medullary thyroid carcinoma or family history of medullary thyroid carcinoma, multiple endocrine neoplasia syndrome);8. Pregnant and lactating women;9. Have used or are using drugs that may affect body weight within 3 months;10. Combined malignant tumor, acute or chronic infectious stage of disease, infectious disease and other chronic wasting disease, mental and psychological disease, drug or other drug abuse; combined with severe cardiopulmonary and cerebral insufficiency, such as respiratory failure, heart failure, myocardial infarction, severe cerebrovascular disease, and so on.

Data and sample collection

Socio-demographic information (name, sex, age, date of birth) was collected from all the study subjects, and detailed inquiries were made about current medical history, past history, family history, behavioral and lifestyle habits (diet, exercise, smoking, alcohol consumption, etc.). Height and weight were measured early in the morning on an empty stomach, after removing shoes and jacket, and BMI was calculated. The patient was instructed to stand in a standing position after urination, with feet 25-30 cm apart, hands naturally hanging down, maintaining normal breathing, and relaxing the trouser belt. The waist circumference was measured at the end of exhalation and inhalation with a soft tape measure around the abdomen through the midpoint of the line between the anterior superior iliac spine and the lower edge of the 12th rib. The examinee was instructed to empty the bladder, rest for 10 minutes to take a sitting position, arms naturally placed on the desktop, the upper arm should be placed at the same level as the heart, the right upper limb was measured 2 times consecutively and then the average value was taken.

Collection of biochemical indexes: Fasting and fasting blood was collected from the subjects and tested in the Laboratory Department of Heji Hospital affiliated to Changzhi Medical College, the indexes included: glucose metabolism indexes: fasting glucose (FBG), fasting C-peptide (FCP), glycosylated hemoglobin (HbAlc), lipid metabolism indexes: total cholesterol (TC), total triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C).

Dietary education: all T2DM patients were provided with diabetes health education and guidance before and after enrollment and during the experiment. Specifically: according to the dietary recommendation standard of medical nutrition therapy, standard weight was calculated according to the formula of adult standard weight: adult standard weight = height (cm) - 105, according to the type of patient's physical activity (bed rest, light physical labor, moderate physical labor, heavy physical labor), combined with the calorie supply for adult diabetic patients recommended by the medical nutrition therapy (kcal/kg of standard body weight), standard calories were calculated, and the proportion of nutrients (carbohydrate calories accounted for 55-65% of total calories, fat calories accounted for 55-65% of total calories) was calculated. Standard calories were calculated, and the proportion of nutrients (55-65% of total calories from carbohydrates, 20-25% of total calories from fat, and 10-20% of total calories from proteins) was carried out to calculate the proportion of each nutrient.

Collection of fecal specimens: fecal samples were collected on the morning of the day they were received, and study participants collected fecal samples in sterile and sealed fecal collection tubes provided by the study team and sealed with the preservation solution that came with the collection tubes. They were also immediately stored in the laboratory in a -80°C refrigerator until analysis.

Sample detection

Blood sample detection methods: FBG was determined by hexokinase assay (Beckman Coulter Automatic Biochemical Analyzer, AU5800, USA); FCP was determined by electrochemiluminescence assay (Roche Electrochemiluminescence Immunoassay Analyzer, Elecsys 2020, Switzerland); HbAlc was determined by high-performance liquid chromatography assay (Burroughs Glycosylated Hemoglobin Assay, BIO- D10, USA); TC was determined by enzymatic method; TG by GPO-POD method; LDL-C by direct method; HDL-C by analytical method; hs-CRP by immunoturbidimetric method; hs-CRP by enzymatic method. D10); TC was measured by enzymatic method; TG was measured by GPO-POD method; LDL-C was measured by direct method; HDL-C was measured by analytical method; hs-CRP was measured by immunoturbidimetric method. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as HOMR-IR=1.5 +FBG (mmol/L)×FCP (nmol/L)/2800.

Methods of gut microbiota analysis: DNA extraction, PCR amplification and sequence analysis: PCR amplification of the V4 region of the 16S rDNA gene was used to prepare sequencing libraries for high-throughput sequencing analysis (Shanghai BaoTeng Biomedical Technology Co., Ltd.). Raw sequences were obtained and screened for clustering of ASVs and species annotation. According to the abundance distribution of ASVs in different samples, we analyzed and compared the α-diversity and β-diversity of the gut microbiota between groups, and observed the community diversity and species richness, and the ecological structure of the gut microbiota. We also performed the analysis of differential gut microbiota between groups, PICRUSt prediction of differential functional metabolic pathways, and so on. Further statistical analyses were performed using the macrogenome mapping software package v2.1.3.

Metabolomics analysis methods: (1) Metabolite quantification: Tandem mass spectrometry coupled with high performance liquid chromatography (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters CorpMilfordMA,USA) was used for the quantification of metabolites in this project. (2) Data processing and analysis: ①The raw data files generated by UPLC-MS/MS were processed using TMBQ software (v1.0, Metabo Profile, Shanghai, China) for peak integration, calibration and quantification of each metabolite. iMAP platform (v1.0, Metabo Profile, Shanghai, China) was used for statistical analysis, including PCA, OPLS-DA, univariate analysis and pathway analysis. ② Mass spectrometry-based quantitative metabolomics referred to the determination of the concentration of a substance in an unknown sample by comparing the sample with a set of standard samples (i.e., calibration curves) of known concentration. ③Multivariate statistical analysis: principal component analysis (PCA). ④ OPLS-DA (Orthogonal Partial Least Squares One Discriminant Analysis) was selected in this study to eliminate noise information not related to grouping and to screen for plausible metabolites that cause differences in grouping. The metabolites can be screened by VIP

scoring through modeling analysis, and the higher the VIP score, the greater the contribution to the grouping. (3) Differential metabolite identification: ① Multi-dimensional statistics: Based on the results of the OPLS-DA model, the volcano plot (Volcanoplot) was used to screen reliable metabolic markers. The thresholds in the Volcanoplot were set as follows: VIP>1. (2) Unidimensional statistics: Unidimensional tests (TTest or Mann-Whitney U Test based on the normality and variance alignment of the data) were used to obtain the differential metabolites between the two groups. (4) Pathway analysis: using selected Pathway-associated metabolite sets (SMPDB) library, Predicted metabolite sets library, the differential metabolites were imported into the iMAP platform for pathway enrichment analysis; hsa library was utilized for pathway analysis.

Statistical analysis

SPSS26.0 statistical data processing software was applied to analyze. Normality test and variance chi-square test were performed for all the measurement data, and the data with normal distribution of measurement data were expressed by mean earth standard deviation (SD), and skewed distribution by Mean (P25-P75). If the data belonged to normal distribution and variance chi-square the independent samples t-test was used between two groups, and ANOVA test was used between multiple groups. If the above conditions were not met, the two groups were compared using the Mann-WhitneyU nonparametric test, multi-group comparisons of the Kruskal-Wallis test; statistical description of the counting data using the rate or the composition of the ratio, and the comparison of groups using the chi-square test. Spearman analysis was used to analyze the correlation between gut microbiota, metabolites and biochemical indicators. Differences were considered statistically significant at $P<0.05$.

Results

Analysis of clinical data, glucose metabolism indexes, and levels of inflammatory factors in T2DM patients

Thirty patients with normal-weight T2DM and 30 patients with obese T2DM were included in this study. We found that T2DM patients did not have statistical differences in sex, age and height ($P>0.05$).The weight, BMI, SBP, waist circumference, FCP, HOMR-IR, and TG in the NNT group did not show significant abnormalities compared with those in the NCP group ($P>0.05$), but DBP, HbAlc, FBG, TC, and LDL-C were all higher than those in the NCP group ($P<0.05$), and HDL-C was significantly lower than that in the NCP group ($P<0.05$).The weight, BMI, DBP, waist circumference, HbAlc, FBG, FCP, HOMR-IR, TC, and LDL-C in the UNT group were higher than that in the NCP group ($P<0.05$), and HDL-C was significantly lower than that in the NCP group ($P<0.001$). Compared with the NNT group, weight, BMI, waist circumference, FCP, and HOMR-IR were elevated in the UNT group ($P<0.05$), and no significant abnormality of lipid metabolism indexes was observed between the two groups ($P>0.05$).In T2DM patients, HbA1c and FBG decreased in the NNT group after metformin treatment ($P<0.05$), while TC, TG, and LDL-C decreased compared to before, but the difference was not statistically significant ($P>0.05$).In the UNT group, BMI, HbA1c, FBG, HOMR-IR, TC, and LDL-C decreased after metformin treatment compared to before ($P<0.05$). Among the drug treatment groups, the concentrations of six inflammatory factors in the MET group were lower than those in the UNT group, with a statistical difference in the comparison of MCP-1, IL-6, and TNF-α ($P<0.05$) (Table 1).

The six indicators that responded to the systemic inflammatory state were higher in the UNT group and the NNT group than in the NCP group, in which the contrast of MCP-1, IL-6, TNF-α, and hs-CRP was significant ($P<0.001$); the contrast of resistin had a statistically significant difference ($P<0.05$), whereas the contrast of CXCL-1 was not statistically significant ($P>0.05$), and the concentration of the six inflammatory factors in the UNT group were all were higher than those in the NNT group, among which IL-6 was statistically different in comparison ($P<0.05$). Among the drug treatment groups, the concentrations of six inflammatory factors in the MET group were lower than those in the UNT group, with a statistical difference in the comparison of MCP-1, IL-6, and TNF-α ($P<0.05$), and the concentrations of six inflammatory factors in the NMT group were lower than those in the NNT group, but the difference did not have a statistical difference ($P>0.05$) (Table 1).

Analysis of the gut microflora diversity of the study subjects

Colony sequencing results: we collected a total of 140 samples from the five groups (Figure 1A), the overlapping ASV data in the Venn diagrams showed a total of 3,189 ASVs in the five groups, and the numbers of ASVs in the NCP, NNT, NMT, UNT and MET groups were 4,925, 4,781, 4,796, 6,585, 5323, respectively. The numbers of ASVs unique to each group were 85, 43, 43, 440, 73, respectively.

α-diversity analysis: the analysis showed that the differences in Shannon index, Simpson index, and Chao1 index among NCP, NNT, NMT, UNT and MET groups were not statistically significant ($P>0.05$) (Figure 1B).

β-diversity analysis: The *PCoA* plot of similarity level between the detected fecal microbial communities based on the *Bray-Curtis* distance showed that the contribution of each component was PC1 = 10.04%, PC2 = 7.96% ($P=0.0404$), and the *PCoA* plot of similarity level among the detected fecal microbial communities based on the *Unweighted Unifrac* distance made to detect the similarity level between fecal microbial communities *PCoA* plot showed that PC1 = 11.1% and PC2 = 6.51% ($P=0.022$). The results showed that the difference in β-diversity reflecting the similarity in the structure of the gut microbiota was statistically significant ($P<0.05$) among the five groups (Figure 1C).

Analysis of differential species between groups: according to the above analysis, the composition and structure of the gut microbiota of

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TABLE 1 Analysis of general data of T2DM patients.

	NCP (n=20)	NNT (n=30)	NMT (n=30)	UNT (n=30)	MET (n=30)	*p*
sex	8/12	13/17	13/17	14/16	14/16	0.264
age	46.20 ± 9.00	46.63 ± 6.90	46.63 ± 6.90	44.45 ± 5.95	44.45 ± 5.95	0.146
height	166.30 ± 7.55	166.98 ± 8.81	166.98 ± 8.81	169.08 ± 8.67	169.08 ± 8.67	0.732
weight	60.00(55.20 68.50)	62.11(55.45 67.65)^{c**}	61.67(55 68)	85.55(79.25 90.08)^{b**}	80.80(75.88 86.)	<0.001
BMI	22.60(61.00 23.22)	22.18(21.25 23.44)^{c**}	22.18(21.55 22.86)	30.16(28.47 31.00)^{b**}	28.15(26.55 29.48)^{e*}	<0.001
waistline	81.09 ± 7.19	80.80 ± 4.03^{c**}	81.04 ± 3.77	95.26 ± 4.78^{b**}	93.42 ± 7.57	<0.001
SBP	116.0(111.0 125.5)	126.5(117 136)	127.57(122 135)	124.92(109 141)	127(110 136)	0.103
DBP	75(71 81)^{a*}	83(74 89)	83(77.75 88)	82.96(72.75 96.75)^{b*}	85(72 88.25)	0.093
HbA1c	5.10(5.05 5.50)^{a**}	8.63(7.3 10.18)	7.00(6.13 7.88)^{d*}	8.82(6.5 10.58)^{b**}	7.50(6.4 8.98)^{e*}	<0.001
FBG	4.97(4.73 5.38)^{a**}	10.65(8.9 13.1)	7.10(7.26 11.33)^{d**}	10.30(7.21 11.61)^{b**}	7.61(6.64 9.49)^{e*}	<0.001
FCP	0.62(0.45 0.77)	0.65(0.44 0.81)^{c*}	0.63(0.45 0.77)	0.98(0.66 1.25)^{b*}	0.89(0.71 1.34)	<0.001
HOM A-IR	2.61(2.27 2.98)	2.91(2.11 3.38)^{c**}	2.97(2.65 4.26)	4.78(3.77 5.81)^{b**}	4.01(3.08 5.45)	<0.001
TC	3.86(1.23 5.07)^{a*}	5.11(4.21 5.84)	4.73(4.18 5.41)	4.94(4.20 5.72)^{b*}	4.05(3.43 4.62)^{e*}	0.003
TG	1.80(1.08 3.40)	2.14(0.96 2.77)	1.79(1.11 2.41)	2.49(1.44 2.88)	1.66(1.30 2.02)	0.415
HDL-C	1.36(1.11 2.02)^{a**}	1.12(0.90 1.38)	1.14(0.91 1.32)	1.03(0.88 1.11)^{b**}	1.00(0.84 1.17)	<0.001
LDL-C	2.27 ± 0.84^{a**}	3.21 ± 0.93	2.98 ± 0.67	3.11 ± 0.84^{b*}	2.42 ± 0.84^{e*}	<0.001
MCP-1	102.78(87.5 112)^{a**}	152.13(124.46 175.90)	149.15 (128.06 165.03)	152.83 (118.25 176.50)^{b**}	128.00 (88.00 140.50)^{e*}	<0.001
IL-6	2.68(2.41 2.87)^{a**}	3.46(3.04 3.45)^{c**}	3.42(3.12 3.69)	4.28(3.42 4.70)^{b**}	3.05(12.61 3.43)^{e*}	<0.001
TNF-α	8.64(5.16 12.01)^{a*}	10.69(9.03 11.73)	10.32(9.24 10.64)	13.26(9.34 14.75)^{b*}	10.85(7.57 12.93)^{e*}	0.001
CXCL-1	34.82(18.98 54.92)	37.13(29.48 41.08)	36.11(29.56 41.57)	39.62(23.48 50.88)	35.70(21.90 45.48)	0.431
resistin	10.64(5.59 12.21)^{a*}	12.34(7.87 13.16)	11.69(4.91 17.37)	16.99(7.97 22.35)^{b*}	14.41(8.05 20.15)	0.064
hs-CRP	0.71(0.35 0.97)^{a**}	1.86(1.14 2.86)	1.65(1.22 2.49)	1.89(1.13 2.43)^{b**}	1.55(0.87 1.90)	<0.001

NCP group, normal control persons; NNT group, normal-weight T2DM patients; NMT group, normal-weight metformin-alone treatment group; UNT group, obese T2DM patients; MET group: obese metformin-alone treatment group. p^a: results of comparison between NNT group and NCP group; p^b: results of comparison between UNT group and NCP group; p^c: results of comparison between NNT group and UNT group; p^d: results of comparison between NNT group and NMT group; p^e: results of comparison between UNT group and MET group; * Represents P <0.05; * * represents P <0.001.

T2DM patients varied between groups. Therefore, in order to find reliable biomarkers, we used LEfSe analysis to assess intergroup differences. The results of the analysis were represented by taxonomic dendrograms and LDA histograms (LDA score >2), which showed the taxonomic hierarchical distribution of the species of the intestinal communities in each group and the species significantly enriched within each group and their level of importance, respectively. As shown in Figures 2A, B, the LEfSe results revealed 16 robust differential biomarkers among the three groups at taxonomic levels above genus level. The biomarkers with LDA scores > 2 in the NCP group were *f_Clostridia_UCG_014*, *g_Clostridia_UCG_014*, *o_Clostridia_ UCG_014*, *g_Eubacterium_xylanophilum_group*; similarly, the NNT group was significantly enriched for *c_Negativicutes*, *g_Megasphaera*, *g_Acidaminococcus*, *p_ Cyanobacteria*, *g_Hungatella*, *g_Eubacterium_hallii_group*; the UNT group screened for *f_Selenomonadaceae*, *g_Megamonas*, *g_Eggerthella*, *o_ Peptostreptococcales_Tissierellales*, *g_Romboutsia*, *f_Eggerthellaceae*.

As shown in Figures 2C, D, *o:Erysipelotrichales, f: Erysipelatoclostridiaceae, g:Intestinibacter were* significantly enriched in the NNT group, *g:Lachnospiraceae_ND3007_ group, g:Anaerostipes, c:Bacilli, c:Clostridia, f:Lachnospiraceae, o: Lachnospirales were enriched in the* NMT group, *g:Eubacterium: eligens_group, g: Eubacterium:xylanophilum_group, o: Peptostreptococcales_Tissierellales were* significantly enriched in the UNT group, *g:Romboutsia, c:Bacteroidia, p:Bacteroidota, o: Bacteroidales were enriched in the* MET group.

Correlation analysis between Differential Bacteria and Clinical Indicators: Figure 3 *g_Intestinibacter* enriched in NNT group was negatively correlated with waist circumference and positively correlated with LDL; *g_Anaerostipes* enriched in NMT group was negatively correlated with HbA1c and positively correlated with LDL; *g_Eubacterium_eligens_group* significantly enriched in the UNT group was positively correlated with LDL, IL-6, and hs-CRP; *Romboutsia* significantly enriched in the MET group was

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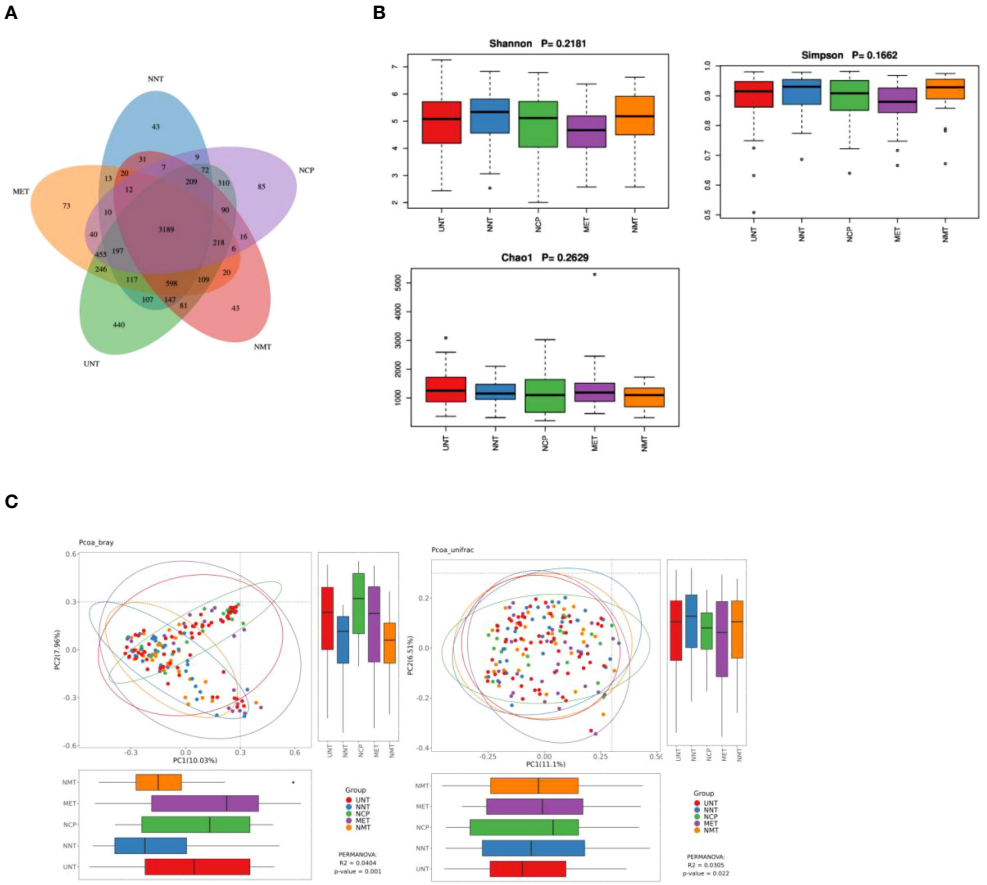

FIGURE 1
Analysis of the gut microflora diversity .**(A)** Venn diagram of ASVs for the five sample groups。 **(B)** Comparison of α-diversity indices. Box plots depicting differences in fecal microbiome diversity among the five groups assessed using the Shannon, Simpson, and Chao1 indices, respectively, are shown. Each box plot represents the median, interquartile range, minimum, and maximum values. **(C)** *PCoA* analysis of five groups of gut microbiota *bray* analysis and *unweighted unifrac*. R-value, used to compare whether there is a difference between different groups; P-value, used to indicate whether there is a significant difference. R-value is between (-1, 1), R-value > 0 indicates that the difference between groups is greater than the difference within groups, R-value < 0 indicates that the difference between groups is less than the difference within groups, R is only a numerical indication of whether there is a difference between the groups and does not provide an indication of significance. The confidence level of the statistical analysis is expressed as P-value, with $P< 0.05$ indicating statistical significance.

positively correlated with HOMA-IR; and *Bacteroidota* was negatively correlated with MCP-1.

Functional prediction: the results of KEGG pathway analysis were shown in Figure 4, and the most different pathways among the four groups were: fatty acid biosynthesis, chlorocyclohexane and chlorobenzene degradation, glycerophospholipid metabolism, and unsaturated fatty acid biosynthesis, and the differences were statistically significant ($P<0.001$).The most different pathways between the MET and NMT groups were: unsaturated fatty acid biosynthesis, chlorocyclohexane and chlorobenzene degradation, binary signaling system, bacterial chemotaxis, and glycerophospholipid metabolism.

Analysis of metabolomics results

Principal component analysis: in this work, targeted metabolomics was applied to explore the intestinal metabolic profile of T2DM patients. As shown in Figure 5A, after principal component analysis, NCP, NNT, NMT, UNT and MET groups showed different distribution trends, with no significant difference in the first principal component ($P>0.05$) and significant difference in the second principal component ($P=0.02$).

Differential metabolite identification: volcano plots showed the relative changes in differential metabolites in the two groups. As shown in Figures 5B–D, 16 differential metabolites were obtained in gut metabolites of normal-weight T2DM patients and 19 differential metabolites were obtained in gut metabolite species of obese T2DM patients compared to healthy controls. 14 differential metabolites were obtained in gut metabolites of obese T2DM patients and normal-weight T2DM patients. The metabolites that were elevated in the normal-weight T2DM group compared to both the obese T2DM group and healthy controls were: dimethylglycine and nordeoxycholic acid; and the metabolites that were decreased in the normal-weight T2DM group compared to both the obese T2DM group and healthy controls were: xylose, ribulose, xylulose, and 4-aminoimauric acid. The box plots in Figure 5E showed the differential metabolites in the gut metabolites of T2DM patients before and after treatment with

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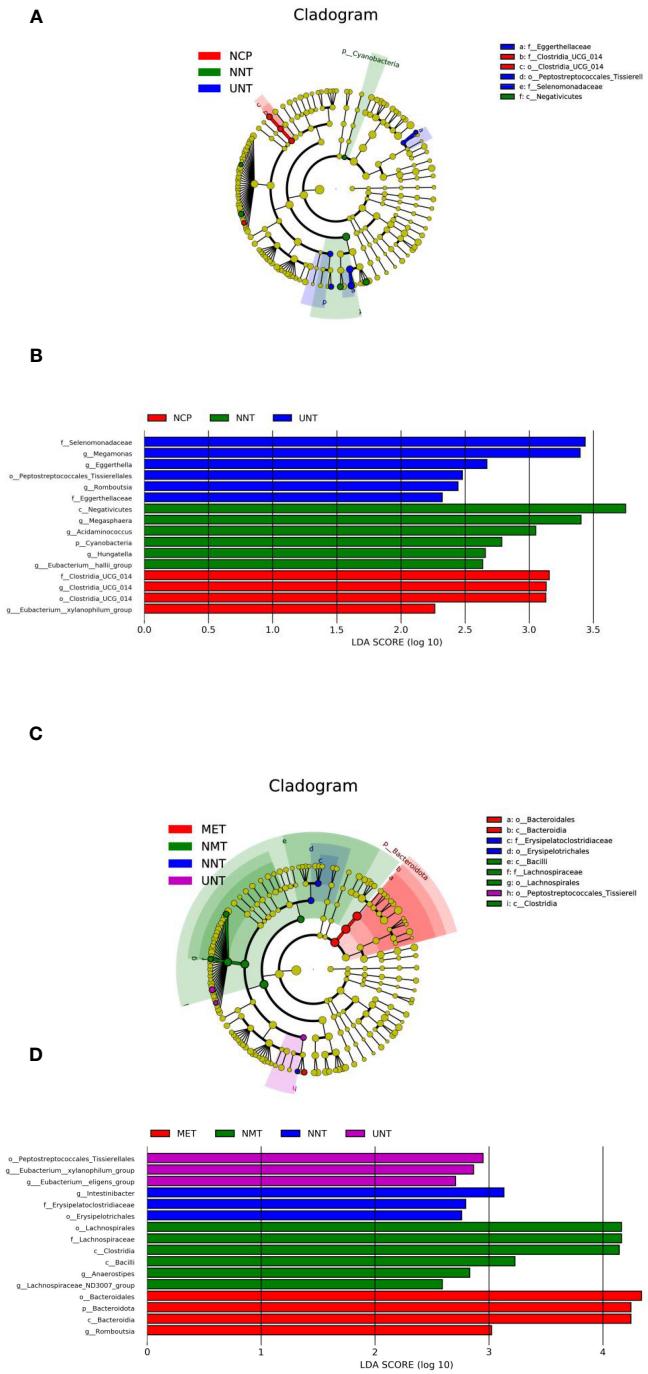

FIGURE 2
Bacterial features most likely explain differences among groups identified by LEfSe based on ASV level. The letter in the former of the name of bacteria indicates different taxa levels (''g'' indicates genus; ''f'' indicates family; ''o'' indicates order;''c'' indicates class; ''p'' indicates phylum). **(A)** Evolutionary branching diagram of LEfse analysis for three sets of samples of NCP, NNT and NMT. **(B)** LDA SCORE (log 10) plots for the three sets of samples of NCP, NNT and NMT. **(C)** Evolutionary branching diagram of LEfse analysis for four sets of samples of NNT, NMT, UNT and MET. **(D)** LDA SCORE (log 10) plots for the four groups of samples of NNT, NMT, UNT and MET.

metformin for four comparisons of groups: the metabolites that were increased in the NNT that showed a more pronounced decrease in the NMT group were: dimethylglycine. the metabolites that were decreased in the NNT that showed a more pronounced increase in the NMT group were: xylose, ribulose, and xylulose.

Analysis of metabolic pathways associated with diabetes mellitus: The major metabolic pathway abnormalities found were Aminoacyl-tRNA biosynthesis (P<0.05); Glycine, serine and threonine metabolism (P<0.05); Valine, leucine and isoleucine biosynthesis (P<0.05); Lysine degradation(P < 0.05); and Pentose and glucuronate interconversions (P<0.05). ((Figure 6A). Amino acids involved in aminoacyl-tRNAs biosynthesis mainly include amino acids such as glycine, serine, methionine, lysine, alanine, isoleucine, leucine, threonine, and tyrosine.(Figure 6B). And xylose,

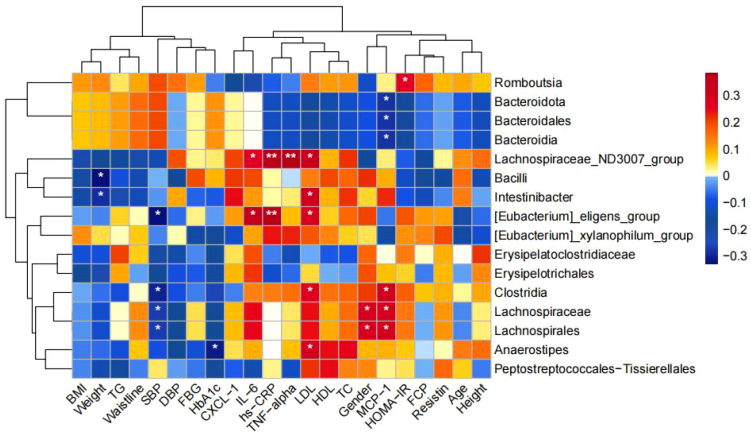

FIGURE 3
Correlation between the four groups comparing differential bacteria and clinical indicators. (*p<0.05, **p<0.01).

ribulose, and xylulose were mainly involved in Pentose and glucuronate interconversions.(Figure 6C).

Correlation between differential metabolites and clinical indicators: the fecal differential metabolites obtained from the comparison of the four groups were subjected to *spearman* correlation analysis with clinical indicators, as shown in Figure 7, and it was found that xylose was positively correlated with BMI, waist circumference, body weight, FCP, HOMA-IR, TG, and negatively correlated with MCP-1; and that ribulose and xylulose were correlated with BMI, waist circumference, body weight, FCP, HOMA-IR, TG were positively correlated and negatively correlated with MCP-1 and HDL-C.

Association between altered gut microbiota and metabolites

As shown in Figure 8, we found that *Intestinibacter* enriched in the NNT group was negatively correlated with Fructose, isoleucine, and Threonine; *g_Anaerostipes* enriched in the NMT group was negatively correlated with Xylose, Ribulose, and Xylulose; and *c_Clostridia* was negatively correlated with sarcosine, Xylose, Ribulose, Xylulose, and isoleucine.

Discussion

T2DM is a common chronic metabolic disease characterized by hyperglycemia due to relative insulin deficiency (12).T2DM accounts for more than 95% of all diabetes mellitus (13), and is an important cause of diabetic complications and high mortality in diabetic patients (14, 15). The risk factors and glucose metabolism characteristics of obesity and diabetes have been widely reported, but the current reports on the characteristics of normal-weight T2DM are still limited. Currently, the physiological mechanisms that promote the progression of T2DM are not fully understood. With the rapid development of sequencing and spectroscopic technologies, the role of gut microbiota and its metabolites in disease progression, it is believed that the gut microbiota and its metabolites are a possible factor influencing the pathogenesis of

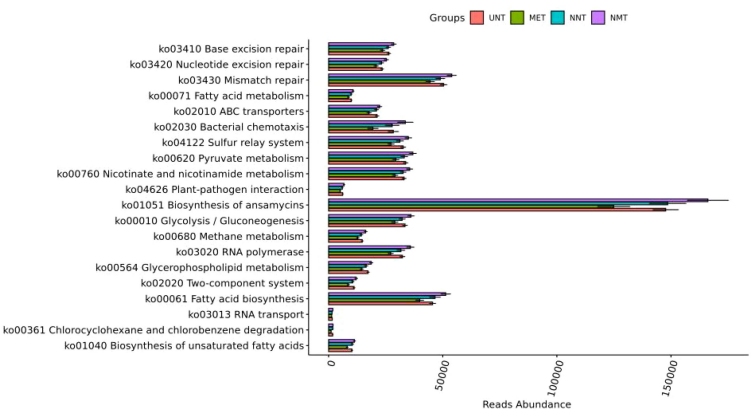

FIGURE 4
Comparison of KEGG pathway analysis results.

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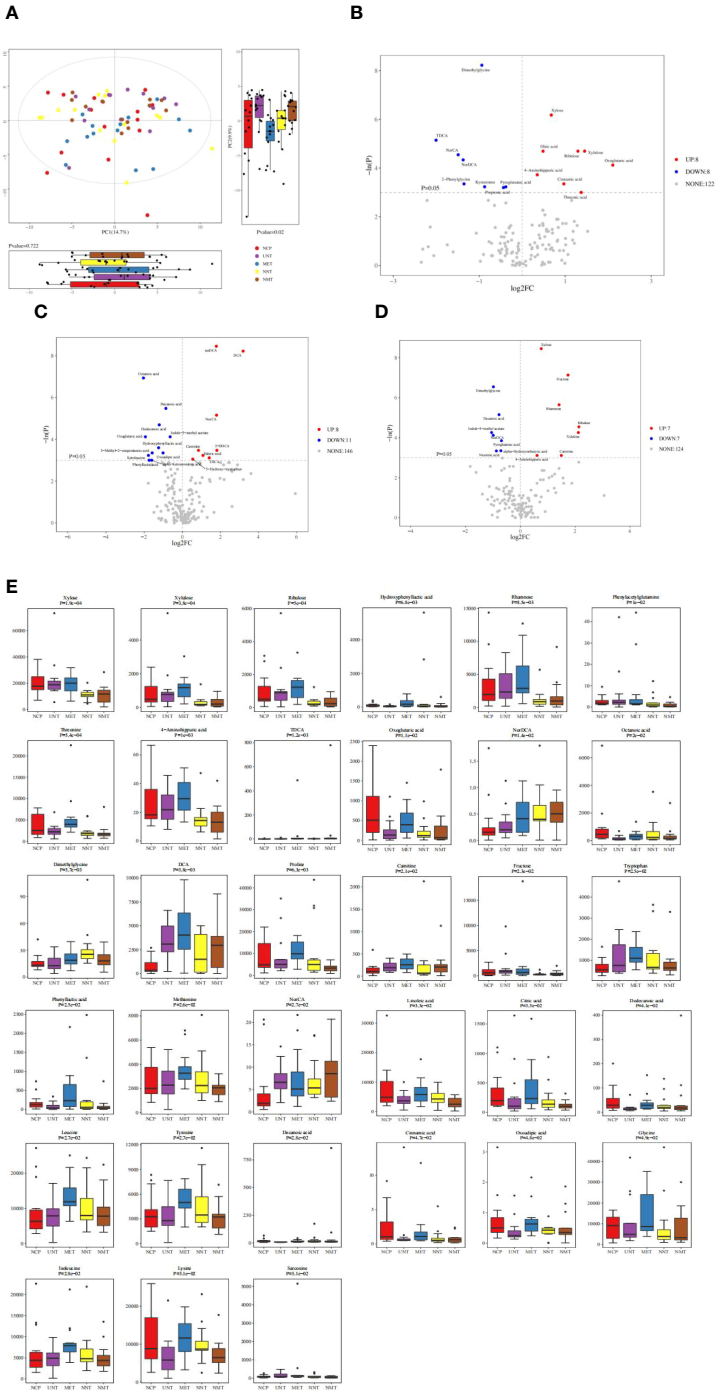

FIGURE 5
Analysis of metabolomics results. **(A)** Principal component analysis graph. The figure shows a plot of the 2D principal component scores of the analyzed samples and a box plot of the corresponding principal component scores. The box plots provide a more intuitive view of the differences in the first and second principal component scores for different groups of samples. Each point in the graph represents a sample, and different colors indicate different groups. The principal components shown in the figure are the combinations of principal components that have the largest distance from each other among all the subgroups. The percentage in parentheses after the principal component represents the overall rate at which that component explains the data. **(B–D)** The vertical dashed line indicates the dotted line corresponding to the FC threshold taken logarithmically and log2FC as the horizontal coordinate; the horizontal dashed line indicates the dotted line corresponding to *P*=0.05 and the corresponding -logeP value as the vertical coordinate. Meanwhile, the points that meet the requirements above the horizontal dashed line and on both sides of the vertical dashed line will be highlighted, **(B)** where the red highlights on the right side indicate the metabolites whose concentration increased, i.e., up-regulated, in the NCP group of the observation group compared to the NNT group of the control group and the blue highlights on the left side indicate the metabolites whose concentration decreased, i.e., down-regulated, in the NCP group of the observation group compared to the NNT group of the control group; **(C)** where the red highlights on the right side indicate the metabolites that were up-regulated by increasing concentrations in the UNT group compared to the control NCP group and the metabolites that were down-regulated by decreasing concentrations in the UNT group compared to the control NCP group. **(D)**The blue highlights on the left side indicate metabolites that were down-regulated by decreasing concentrations in the UNT group compared to the control NNT group; the gray dots indicate metabolites that did not meet the requirements of the set threshold. **(E)** Comparison of differential metabolites among the five groups.

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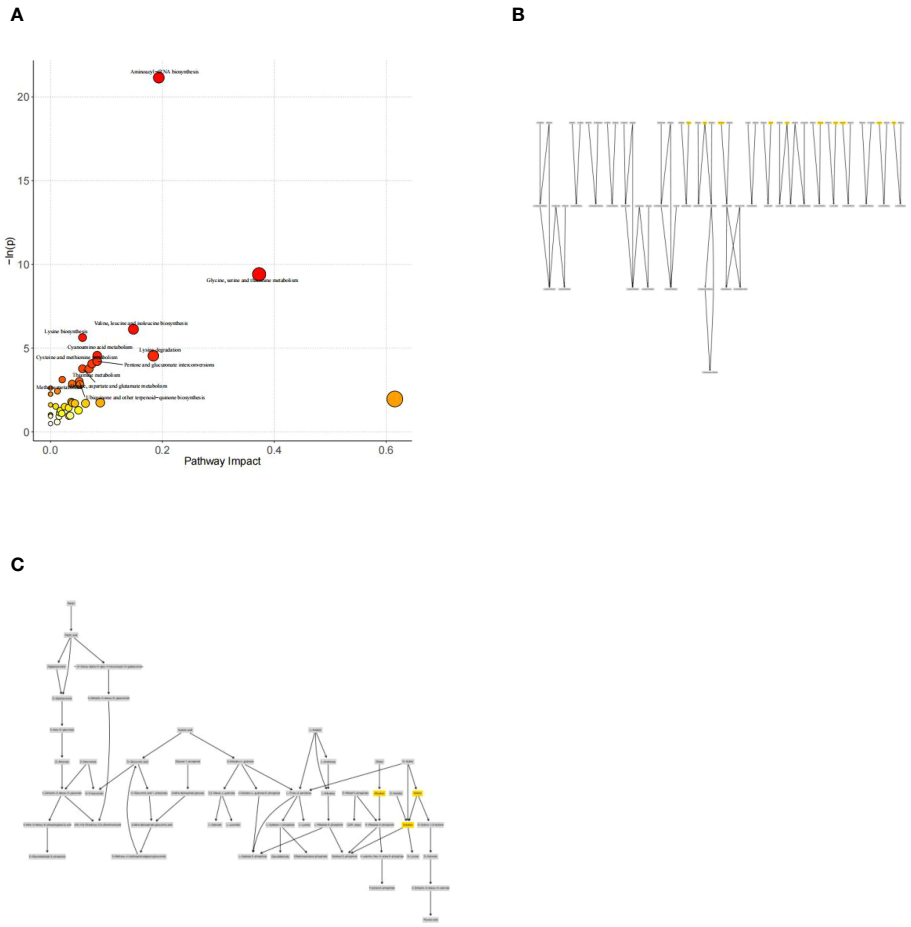

FIGURE 6
(A) Metabolic pathway and influence analysis. Each circle corresponds to a metabolic pathway, the horizontal coordinate indicates the degree of pathway impact, the size of the circle is related to the pathway impact of the pathway, the larger the Impact value the larger the circle, the vertical coordinate indicates the negative logarithm of the P-value obtained from the enrichment analysis of the pathway, and the change of the yellow-red color of the point is positively related to the negative logarithm of the P-value of the pathway change. Correlation. Pathways with $P<0.05$ are labeled with their names in the figure, and pathways that do not meet the above conditions are not labeled with their names in the figure. **(B)** Network diagram for pathway analysis of aminoacyl-tRNAs biosynthesis. **(C)** Network diagram for pathway analysis of Pentose and glucuronate interconversions.

metabolic diseases. Therefore, therapeutic and preventive strategies by regulating the gut microbiota and metabolites in type 2 diabetes mellitus patients are well worth researching and exploring. Current guidelines at home and abroad recommend metformin as a first-line therapeutic agent for T2DM (16), which has the ability to regulate glycolipid disorders, antioxidant and anti-inflammatory effects, modulate glycolipid metabolism and improve insulin resistance by reducing oxidation and inflammation. The presence of dysbiosis in patients with T2DM at first diagnosis is mainly characterized by an increase in the overall activity of the gut microbiota and, at the same time, significant changes in the proportions of some specific genera (17). Therefore, exploring and discovering the effects of metformin on glucose metabolism, lipid metabolism and gut microbiota and their metabolites is important for the prevention and treatment of diabetes. Previous animal experiments and clinical trials had shown that metformin can affect the gut microbiota and its metabolites (6, 18, 19), but studies had mostly focused on obese patients. In contrast, we compared the effects of metformin normal-weight T2DM gut microbiota. In this study, we observed the characteristics of gut microbiota and changes in metabolite fractions in normal-weight T2DM patients by 16S rDNA gene sequencing technology and high performance liquid chromatography-mass spectrometry-targeted metabolomics, and we explored the effects of gut microbiota and their metabolites on glucose and lipid metabolism of the organism after metformin treatment, which can provide a basis for further revealing the relationship between diabetes mellitus and gut microbiota and for guiding the normal-weight T2DM patients with provide the basis for precise treatment program.

Our study found that HbA1c, FBG, FCP, HOMA-IR, TC, LDL-C, were higher and HDL-C was lower in patients with T2DM than in the healthy population, and that weight, BMI, and waist circumference were significantly lower in the healthy population and in the normal-weight T2DM patients than in the obese T2DM patients. Meanwhile, we found that the treatment of T2DM patients with metformin could improve the glycolipid metabolism, but the

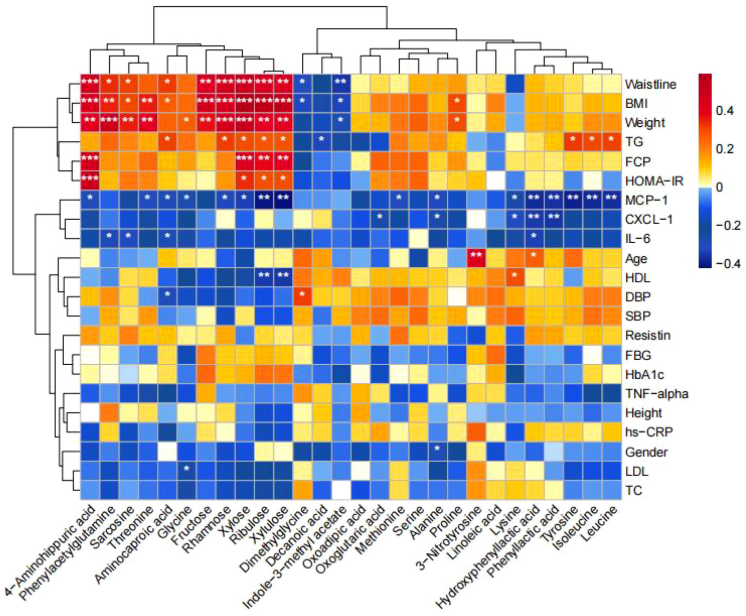

FIGURE 7
Correlation between differential metabolites and clinical indicators. Heatmaps show the correlation between differential metabolites and clinical indicators. Red color represents positive correlation and blue color represents negative correlation. The darker the color, the stronger the correlation. (*p<0.05, **p<0.01, ***p<0.001).

recovery of glycolipid metabolism was more obvious in obese T2DM patients, which may be due to the fact that no weight loss is required for normal-weight T2DM patients and that there exists stricter dietary and exercise interventions for obese T2DM patients. We also compared six inflammatory indicators: MCP-1, IL-6, TNF-α, CXCL-1, Resistin, and hs-CRP were higher in T2DM patients than in the healthy population, but the concentrations of all six inflammatory factors were lower in normal-weight T2DM patients than in obese T2DM patients. This may be related to the fact that both obesity and diabetes are a low-grade inflammatory state, and the degree of inflammation is increased when the two are present in combination. This is consistent with the results reported in the current study (20).Meanwhile, we also compared the six inflammatory indicators in T2DM patients after metformin treatment and found that all of them showed a decreasing trend, which suggests that the reduction of the inflammatory state in the body of T2DM patients after drug treatment may be related to the recovery of their blood glucose level and pancreatic islet function.

We sequenced samples for 16S rDNA gene sequencing. The diversity of the gut microbiota of normal-weight T2DM patients was verified by α and β diversity analysis. The data showed that species diversity and community richness were higher in normal-

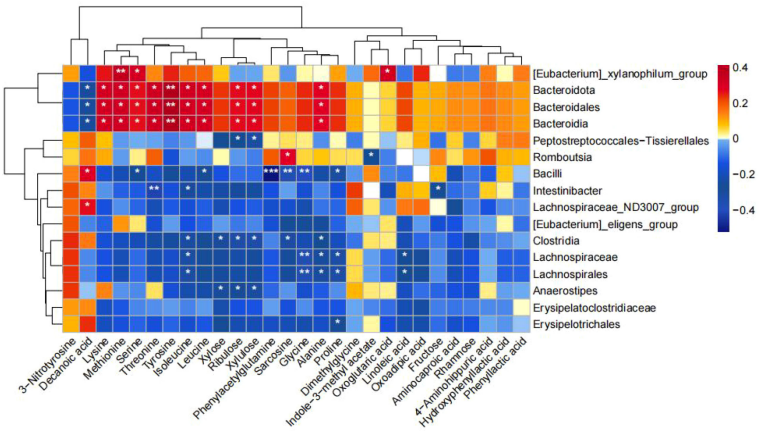

FIGURE 8
Correlation between changes in fecal metabolites and changes in gut bacteria abundance. Heatmap showing the correlation between changes in fecal metabolite concentrations and changes in the relative abundance of enteric bacteria. Red color represents positive correlation and blue color represents negative correlation. Darker colors indicate stronger correlations. (*p<0.05, **p<0.01, ***p<0.001).

weight T2DM patients than in the healthy population, and the overall structure was altered, but not differentially. To further assess the precise changes in the gut microbiota, we performed LefSe to analyze the differential bacteria between groups and found that normal-weight T2DM patients were enriched in *c_Negativicutes*, *g_Megasphaera*, and *g_Acidaminococcus*, and obese T2DM patients were enriched in the gut microbiota in *g_Megamonas*, *g_Eggerthella*, *g_Romboutsia*, and *f_Eggerthellaceae* were higher than those of the healthy population, while *Clostridia* were lower than those of the healthy population. *Acidaminococcus* belongs to the order *Negativicutes* and *Firmicutes*, with glutamate as the main energy source. *Acidaminococcus* has been positively associated with T2DM risk factors, such as fasting blood glucose and HbA1c (21), and has shown a significant positive correlation with cholesterol and triglyceride levels, suggesting that it may be involved in fatty acid metabolism. *Megasphaera*, also belonging to Firmicutes, ferment mainly fructose and lactic acid, and a previous macro-genomic study found higher abundance of *Megasphaera* and lower abundance of *Clostridia* in patients with T2DM and pre-diabetes compared to metabolically healthy controls (22), which is consistent with our study, whereas *Clostridia* is recognized as a probiotic associated with tryptophan metabolism in mice with colitis, and may mediate intestinal barrier disruption, colon inflammation and amelioration of pathological phenotypes (23). In conclusion, our study corroborated the previous characterization of the gut microbiota in the T2DM population, but we also found that there are differences in the changes of gut microbiota *in vivo* for the first diagnosis of T2DM, suggesting that attention needs to be paid to bacterial duality and dynamic changes in future studies. We tested the intestinal microecological balance of the gut microbiota after metformin intervention and compared it with that at baseline, and the data showed that, although it was more similar in terms of community diversity and species richness, the community diversity and species richness were restored after metformin intervention compared with the previous one, which was closer to that of healthy population. This shows that metformin can improve intestinal dysbiosis, which indicates the rationality of clinical glucose lowering for T2DM from the perspective of gut microbiota, which is also a strong proof of the guidelines' heavy recommendation of the first-line drug. Meanwhile, in the analysis of differential bacteria between groups, we found that normal-weight T2DM patients were enriched in the metformin-treated group with one kind of bacterium, *g_Anaerostipes*, which was negatively correlated with HbA1c and positively correlated with LDL-C as suggested by the spearman's correlation with the clinical indexes. The genus *Anaerostipes* is one of the most enriched taxa in the healthy microbiota and one of the butyric acid producing and is one of the most efficient lactic acid consuming bacteria in the human gut microbiota. The abundance of *Anaerostipes* has been reported to be significantly lower in African and European T2DM patients (24, 25). More than half of *Anaerostipes* are able to use inositol as the sole source of carbon and energy and can convert dietary inositol to propionate (26), which has been suggested to have an indirect effect on diabetes by lowering adipogenesis and serum cholesterol levels, thereby decreasing the risk of diabetes development (27). In contrast, in obese T2DM patients, *Romboutsia*, enriched after metformin treatment, was positively correlated with HOMA-IR; *Bacteroidota* was negatively correlated with MCP-1. This suggests that metformin may act through different gut microbiota in normal-weight versus obese T2DM. In summary, we speculated that *Anaerostipes* was a beneficial bacterial genus that plays a role in glycemic dyslipidemia and anti-inflammation, and we can use this as a target for further animal experiments for validation in the future.

Our correlation analysis of gut microbiota with clinical biochemical indices revealed that *Megasphaera* enriched in normal-weight T2DM was positively correlated with FBG, HbA1c, hs-CRP, and MCP-1, *Negativicutes* was positively correlated with FBG and HbA1c, and *Acidaminococcus* was negatively correlated with HDL-C. *Megamonas* enriched in obese T2DM patients was positively correlated with BMI, waist circumference, and HOMA-IR; *Eggerthella* was positively correlated with TNF-α, hs-CRP, resistin, IL-6, and LDL-C. *Clostridia_UCG_014* enriched in healthy controls was negatively correlated with FBG, HbA1c, IL-6, and MCP-1. Consistent with previous studies, this further suggests that gut microbiota may play an important role in the disorders of glycolipid metabolism and inflammatory effects in T2DM patients.

We also observed trends in metabolic pathways: a decrease in aminoacyl tRNA biosynthesis, metabolism of glycine, serine, and threonine, biosynthesis of valine, leucine, and isoleucine, and lysine degradation pathways. Aminoacyl tRNA biosynthesis is involved in the synthesis of amino acids as well as in a variety of metabolic processes such as protein synthesis, hormone synthesis, and glycolipid metabolism (28).Roas et al. found a significant enrichment of metabolites associated with aminoacyl-tRNA biosynthesis after the use of metformin (29), and in our study we found that the metabolism of a wide variety of amino acids centered on aminoacyl- tRNA biosynthesis mainly including amino acids such as glycine, serine, threonine, methionine, lysine, alanine, isoleucine, leucine, and tyrosine, and that a decrease in the metabolic pathways of glycine, serine, and threonine indicated an increase in the levels of glycine, serine, and threonine, which was in agreement with the previous study (30), in which glycine, serine, and threonine were associated with an improvement in insulin sensitivity (31). Previous studies have shown that changes in plasma glycine may be one of the biomarkers of T2DM (32), and Chen et al. found in their study that insulin secretion was higher in diabetic rats taking glycine compared to diabetic rats not taking glycine (33). It was also found that glycine improved the microstructure of pancreatic β-cells and increased the number of mature insulin-secreting granules. Serine is a non-essential amino acid that plays a role in the metabolism of fats and fatty acids and the growth of muscles, and can lower cholesterol levels, helping to prevent diseases such as hypertension and atherosclerosis. Threonine is an essential amino acid that promotes protein synthesis, maintains cell function, promotes liver metabolism, and improves liver detoxification to a certain extent. While metformin can inhibit gluconeogenesis, its mechanism may rely on the AMPK-dependent pathway (34), it was found that metformin can promote the phosphorylation of serine/threonine kinase 11, which phosphorylates T172 on the α1 subunit of AMPK and activates

AMPK and inhibits the adverse effects of hepatic protein kinase B1 on metformin. AMPK Activation triggers cAMP catabolism to reduce glucagon stimulated cAMP and PKA signaling. cAMP and PKA signaling is diminished, glycogen synthesis is enhanced, and gluconeogenesis is inhibited. Valine, leucine, and isoleucine are branched-chain amino acids, and Huda et al. found that insulin-resistant patients exhibit abundant biosynthesis of branched-chain amino acids and were found to lack the genes encoding bacterial inward transporter proteins for these specific amino acids. Phosphorylation of insulin receptor substrate-1 on serine residues by stimulating rapamycin and its downstream effector mTOR/S6 kinase interferes with insulin signaling (35). Therefore, it can be assumed that amino acids play an important role in glucose homeostasis, and supplementation of amino acids, a metabolite, can improve glucose tolerance, and, metformin may regulate the disorders of glucose-lipid metabolism in the T2DM population through amino acid-related pathways, but the mechanisms of these metabolic pathways associated with the hypoglycemic effects of metformin remain to be further investigated. Fecal metabolomics reveals that metformin treatment reverses metabolic abnormalities in normal-weight or obese T2DM patients. Metformin treatment upregulated metabolites that were decreased in normal-weight T2DM patients: xylose, ribulose, and xylulose, and their correlation with clinical indicators was found to be positively correlated with BMI, waist circumference, and body weight in the correlation study. And the metabolites up-regulated in obese T2DM patients were: octanoic acid, decanoic acid, dodecanoic acid, and decanoic acid was negatively correlated with TG. In order to further clarify the association between gut microbiota and these metabolites, we continued the *spearman* analysis of gut microbiota with metabolites, and we found that *Anaerostipes* were negatively correlated with xylose, ribulose, and xylulose; and *Clostridia* were negatively correlated with sarcosine, xylose, ribulose, xylulose, and isoleucine. This suggests that metformin may exert its effects in normal-weight versus obese T2DM patients possibly through different gut microbiota and metabolites.

Gut microorganisms interact with the host by producing different metabolites. Therefore, we used UPLC-MS/MS to quantify targeted Q200 macro-metabolomics in fecal samples. The metabolites that were significantly reduced in normal-weight T2DM patients compared to obese T2DM and healthy controls consisted of pentose, glucuronic acid metabolism, mainly xylose, ribulose, and xylulose. In contrast, the metabolites that were significantly reduced in the intestines of obese T2DM patients compared to normal-weight T2DM and healthy controls were mainly composed of lipid metabolism, such as octanoic acid, dodecanoic acid, and decanoic acid. On the contrary, the metabolites that were significantly increased in obese T2DM patients compared to normal-weight T2DM and healthy controls consisted mainly of bile acid metabolism, mainly DCA, NorCA. xylose, ribulose and xylulose all belong to pentose, while pentose and glucuronic acid are two common glycoconjugates, which are important for research in biochemistry, medicine, and other fields. Xylose is a component of xylan with anti-bacterial and anti-mold properties; it helps to inhibit the growth and reproduction of harmful microbiota in the gut and enhances the growth of beneficial microbiota in the gut, such as *Bifidobacterium*, which has significant health benefits, including improved intestinal permeability, which leads to lower circulating levels of endotoxins and reduced systemic inflammation. This has been linked to improved glucose tolerance and glucose-induced insulin secretion in the host and reduced inflammation (36). Most importantly, xylose also has hypoglycemic properties that help promote insulin secretion in the body, reduce blood glucose levels and control the onset or progression of metabolic diseases such as diabetes. Ribulose is a monosaccharide with a pentose structure corresponding to ribose, which can appear in the reductive pentose phosphate cycle in photosynthesis. Su Tao et al. found that T2DM not only had significantly higher urinary glucose concentrations than normal controls, but also significantly higher urinary pentose concentrations, suggesting that not only glucose metabolism but also pentose metabolism was abnormal in T2DM (37). It had also been found that intravenous pentose could significantly increase serum insulin level and maintain it for a long period of time, and its effect of lowering blood glucose was only significant after 5 minutes of intravenous pentose, and the effect of lowering blood glucose gradually disappeared with the prolongation of time (38). In the present study, we found that the major metabolic pathways of normal-weight T2DM distinguishing healthy populations by pathway analysis of differential metabolites in the hsa library were: pentose, glucuronide interconversion, fatty acid biosynthesis, starch and sucrose metabolism. We also identified pentose as part of the potential biomarkers associated with the pentose phosphate pathway. Thus, pentose promotes both energy metabolism and improves energy metabolism in ischemic and hypoxic cells, as well as promotes insulin production and lowers blood glucose. Octanoic acid, dodecanoic acid, and decanoic acid belong to medium-chain fatty acids, and related studies have also found that medium-chain fatty acids can reduce T2DM-induced hyperlipidemia, insulin resistance, oxidative stress, and inflammatory response, repair liver function damage, and promote glycogen synthesis. It also activates PI3K/AKT/GLUT-2 signaling pathway, promotes glucose metabolism gene expression and maintains glucose homeostasis. And the correlation study with clinical indicators found that Dimethylglycine was positively correlated with age, DBP, LDL-C, TC, hs-CRP, and NorDCA was positively correlated with FBG, HbA1c, HOMA-IR, DBP, LDL-C, TC, IL-6, resistin, hs-CRP; xylose was positively correlated with FBG, HbA1c, HOMA-IR, DBP, LDL-C, TC, IL-6, resistin, hs-CRP. Xylose was positively correlated with waist circumference and negatively correlated with age. Decanoic acid was negatively correlated with waist circumference, BMI, TG, and positively correlated with HDL-C; Octanoic acid was negatively correlated with waist circumference, BMI, TG, FBG, HbA1c, IL-6, and positively correlated with HDL-C; and Dodecanoic acid was negatively correlated with waist circumference, BMI, TG, FBG, HbA1c, and positively correlated with HDL-C. This all suggests that changes in intestinal metabolites may all play an important role in the development of metabolic diseases such as T2DM, and that different metabolites may play a role in obesity and normal-weight T2DM.

In conclusion, we used 16S rDNA sequencing technology and Q200-targeted macro-metabolomics approach to understand

changes in gut microbiota and its metabolite fractions and functional metabolic pathways in patients with T2DM compared to healthy controls. It was determined that there were significant overlaps and differences in the composition and functional characteristics of the gut microbiota in the normal-weight T2DM group, the obese T2DM group and the healthy control group. On this basis, we found that *c_Negativicutes, g_Megasphaera*, xylose, ribulose and xylulose play important roles in the development of normal-weight T2DM, and in obese T2DM, *g_Megamonas, g_Eggerthella, g_Romboutsia*, decanoic acid, octanoic acid, and dodecanoic acid may be specific microbiota and metabolites that play important roles in the pathogenesis of obese T2DM. Similarly, we found that *Anaerostipes*/xylose/ribulose/xylulose may play an important role in the treatment of normal-weight T2DM with metformin by improving glycemic lipids and reducing inflammation. However, the related microbiota and metabolites have been less studied in the population, especially in normal-weight type 2 diabetes mellitus, which deserves to be verified in future animal experiments as well as related population intervention trials.

This study has some limitations. First, the small sample size of this study cannot represent the relevant changes of microbiota and metabolites in T2DM patients at different BMI, so further large-scale longitudinal, interventional and multicenter studies are needed in the future. Second, in recent years, several papers have reported the effects of diet and exercise on gut microbiota and metabolites, and although we provided health education to our patients, we were not able to completely avoid this influencing factor due to the fact that no weight loss is required for normal-weight T2DM patients and that patients have limitations such as self-control and work and other related factors, which may have an impact on our results. Finally, we selected T2DM patients with a short course of the disease, and our results are not applicable to T2DM patients with a longer course of the disease.

The strength of this study is that we successfully applied 16S rDNA sequencing technology and Q200 quantitative macrometabolomics to reveal the characteristics of intestinal microecological dysregulation and metabolic disorders in patients with normal-weight T2DM, and we also found that metformin could intervene in normal-weight T2DM by reversing the abnormalities of pentose, amino acids, and other related metabolisms and by modulating interactions between metabolites and gut microbiota. In conclusion, the We selected normal-weight T2DM patients with short disease duration and included obese T2DM patients to analyze the role of weight in the gut microbiota and its metabolites in T2DM patients, to further understand the gut microbiome-metabolome interactions in normal-weight T2DM patients and to systematically elucidate the pathogenesis of T2DM from the perspective of the host microbial metabolic axis and metformin's possible Therapeutic mechanisms.

Conclusion

In summary, we analyzed and compared the microbiota characteristics of normal-weight T2DM, and we hypothesized that the gut microbiota affect the host through metabolites, which provides further understanding of microbiome-metabolome interactions in T2DM, and may be useful for the future diagnosis and treatment of T2DM, adding to this field. In addition, we combined 16S rDNA sequencing technology and Q200 quantitative macrometabolomics and found that metformin treatment normalized the diversity and abundance of the gut microbiota and its metabolites in normal-weight type 2 diabetic patients. It may be related to *Anaerostipes*-xylose/ribose/xylulose. Correlation analysis between gut bacteria and metabolites not only highlights their relationship, but also helps to explain the pathogenesis of normal-weight T2DM and potential mechanisms of drug therapy.

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: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1133738.

Ethics statement

The studies involving humans were approved by Changzhi Medical College Affiliated Heji Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XN: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. YW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. LH: Data curation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing. PG: Investigation, Writing – review & editing. SZ: Investigation, Validation, Writing – review & editing. YS: Conceptualization, Methodology, Supervision, Writing – review & editing. MJ: Conceptualization, Methodology, 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.

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The gut microbiota changed by ketogenic diets contribute to glucose intolerance rather than lipid accumulation

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The ketogenic diet (KD) is a popular option for managing body weight, though its influence on glucose and lipid metabolism was still inconclusive. Gut microbiota is modulated by dietary pattens and has been associated with the changes of metabolic homeostasis induced by KD. Here, we found that two types of KDs, KD1 (8.8% carbohydrate, 73.4% fat, 17.9% protein, 5.7 kcal/g) and KD2 (0.4% carbohydrate, 93.2% fat, 6.4% protein, 6.7 kcal/g), induced changes of gut microbiota and its metabolites, contributing to glucose intolerance but not lipid accumulation in mice. Following a 2-week intervention with KDs, mice fed on KD1 displayed symptoms related to obesity, whereas KD2-fed mice exhibited a decrease in body weight but had severe hepatic lipid accumulation and abnormal fatty acid metabolism, while both KDs led to significant glucose intolerance. Compared to the mice fed on a standard chow diet, the conventional mice fed on both KD1 and KD2 had significant shifted gut microbiota, lower levels of short chain fatty acids (SCFAs) and composition alteration of cecal bile acids. By using an antibiotic cocktail (ABX) to deplete most of the gut microbiota in mice, we found the disturbances induced by KDs in lipid metabolism were similar in the ABX-treated mice to their conventional companions, but the disturbances in glucose metabolism were absent in the ABX-treated mice. In conclusion, these findings suggest that ketogenic diets disrupted glucose and lipid metabolism, at least in mice, and highlight the gut microbial culprits associated with KD induced glucose intolerance rather than lipid accumulation.

KEYWORDS

ketogenic diet, gut microbiota, glucose and lipid metabolism, SCFA, bile acid

1 Introduction

The ketogenic diet (KD) is a high-fat, low-carbohydrate and moderate protein diet (1, 2). By severely restricting carbohydrates and increasing fat content, the ketogenic diet shifts the

body's primary energy source from glucose to ketone bodies (KB), which are intermediate products produced during fatty acid oxidation in the liver (3–6). Because of this, ketogenic diets are popular in weight loss management (7). Despite its effectiveness in promoting weight loss, numerous studies in both human and animal models have raised concerns regarding the adverse effects of KD on glucose and lipid metabolism. In human studies, consumption of KD has been associated with unfavorable alterations in lipid profiles, including elevated levels of LDL cholesterol and triglycerides (8). Additionally, KD interventions have been shown to induce transient increases in postprandial glucose levels, raising concerns about its impact on glucose metabolism (9). These findings are further supported by animal studies, which have demonstrated the development of hepatic insulin resistance induced by KD (10). However, the mechanism by which KDs influence glucose and lipid metabolism remains elusive.

Previous studies have highlighted the potential impact of the ketogenic diet (KD) on gut microbiota composition and bacterial metabolites (11–14). A systematic review reveals that KD can lead to a decrease in the abundance of *Bifidobacterium* and butyrate-producing bacteria belonging in Firmicutes, resulting in lowered levels of SCFAs, potentially promoting obesity, gastrointestinal disorders, and T2D (15). Our previous research also showed significant alterations in the composition and content of SCFAs, bile acids, and tryptophan metabolites in mice fed with KDs, which were closely related to the disorders of glucose and lipid metabolism (16). Despite these observations, our understanding of the mechanistic pathways by which gut microbiota and microbe-associated metabolites influence the metabolic effects of KD remains limited. Therefore, further investigation is warranted to elucidate the complex interplay between KD, gut microbiota, and host metabolism.

In this study, we aimed to investigate the impact of ketogenic diets (KDs) on glucose and lipid metabolism and elucidate the role of gut microbiota in the metabolic disturbances induced by KDs. To achieve this, we employed two types of KDs: KD1, typically used in clinical trials (7), and KD2, commonly used in mice trials (17). Through short-term dietary interventions in both specific pathogen-free (SPF) mice and microbiota-depleted mice, we sought to explore the contribution of gut microbiota to KD-induced metabolic alterations. Our findings revealed that both KDs induced glucose and lipid metabolism disorders, but only glucose intolerance induced by KDs depended on gut microbiota. This study provides future research directions for exploring the mechanisms underlying the effects of KD on host glucose and lipid metabolism, and it emphasizes the critical role of gut microbiota in the mechanisms of KD.

2 Materials and methods

2.1 Animal trials

Five-week-old male C57BL/6 mice were obtained from SLAC Inc. (Shanghai, China) and then kept under the specific pathogen-free (SPF) conditions at the animal facility of Shanghai Jiao Tong University, Shanghai, China (12h light/dark phase cycle, temperature at 22°C ± 3°C). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University (No. A2021043).

2.1.1 Trial 1

After one-week of acclimation, the mice were provided normal chow (NC, 3.9 kcal/g, with 65.6% of calories from carbohydrate, 16.3% from fat, and 18% from protein) and pure drinking water for 4 weeks. Then the mice were randomly assigned to 3 groups: (1) NC group (n = 10), control mice fed normal chow; (2) KD1 group (n = 10), mice fed a ketogenic diet commonly used in clinical studies (KD1, 5.7 kcal/g, with 8.8% of calories from carbohydrate, 73.4% from fat, and 17.9% from protein); (3) KD2 group (n = 10), mice fed a ketogenic diet commonly used in animal trials (KD2, 6.7 kcal/g, with 0.4% of calories from carbohydrate, 93.2% from fat, 6.4% from protein). All mice were allowed ad libitum access to water and freshly prepared food every day for 2 weeks. The body weight of the mice was measured every day.

2.1.2 Trial 2

After one-week of acclimation, a cocktail of antibiotics (0.5 g/L of vancomycin, 1 g/L of ampicillin, 1 g/L of neomycin, 1 g/L of metronidazole) was introduced in drinking water for 4 weeks. The mice were then randomly assigned to 3 groups: (1) ABX + NC group (n = 10), fed normal chow (NC). (2) ABX + KD1 group (n = 10), fed KD1. (3) ABX + KD2 group (n=10), fed KD2. The mice were allowed ad libitum access to water and food for 2 weeks. During the 2 weeks, all groups continued to have the antibiotic cocktail in their drinking water. All mice were fed freshly prepared diets every day and the body weight were measured every day.

Both trials used the same batch of mice and started at the same time. All diets involved were produced by SYSE Ltd., Changzhou, China. The formulas of these diets are shown in Supplementary Table S1. Fecal samples were collected at week 2 and stored at -80°C for gut microbiota analysis. All mice were fasted for 6 h before sampling. Blood samples were collected from the orbital vascular plexus. Serum samples were isolated from blood samples after centrifugation for 15 min at 4°C at 3,000 g and then stored at -80°C. Liver, pancreas, adipose tissues (epididymal, retroperitoneal, perirenal, mesenteric), cecum contents and colon contents were weighed and collected immediately and stored at -80°C or in 4% paraformaldehyde for further analysis.

2.2 Oral glucose tolerance test

All mice were fasted for 6 h and then administered glucose (2 g/kg body weight) through oral gavage. The blood glucose concentrations were measured from the tip of the tail vein at 0 (the fasting glucose) and 15, 30, 60, 90, and 120 min after glucose administration using a blood glucose meter (Accu-Chek Performa, Roche, USA). Blood samples were collected from the tail vein into tubes at 0, 15, and 60 min after glucose administration.

2.3 Serum insulin measurement

Enzyme-linked immunosorbent assays (ELISAs) were used to measure the serum insulin (90080, Chrystal Chem, USA) in accordance with the manufacturer's instructions.

2.4 Blood ketone measurement

After 6 h of fasting, blood ketone (β-hydroxybutyrate) levels were measured in blood samples collected from the tip of the tail vein with a blood ketone meter (FreeStyle Optium Neo, Abbott, USA).

2.5 Liver triglycerides measurement

Frozen liver samples were homogenized in a corresponding volume (w:v = 1:9) of absolute ethanol. The supernatant was collected after centrifugation for 25 min at 2,000×g and 4°C. Triglycerides in the homogenized tissue were quantified using colorimetric kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.6 Serum ALT and AST measurement

The concentrations of ALT and AST in serum were quantified with kits (C009-2-1 and C010-2-1, respectively) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.7 Histopathology of epididymal fat, perirenal fat, liver and pancreas

For epididymal fat and liver, the fresh epididymal fat pad and the largest lobe of the liver were fixed with 4% paraformaldehyde solution for 48 hours. After the tissues were embedded in paraffin, the sections about 4 μm thickness were stained with hematoxylin and eosin (H&E). The size of adipocytes and the area of liver steatosis were calculated using Image-Pro Plus v6.0 (Media. Cybernetics Inc., Silver Springs, MD). The specific steps were as follows: for adipose tissue, the number of adipocytes in three pictures (400× magnification) was counted for each sample, and the average adipocyte size was calculated based on the total area (cross-sectional area) divided by the number of adipocytes. For liver tissue, a histological score (NAFLD Score) was performed according to the method described by David E et al. (18).

For perirenal adipose tissue, the right fat pad was directly frozen in liquid nitrogen during dissection, and then stored at -80°C. The frozen tissue was subsequently dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) (Wuhan Seville Biotechnology Co., Ltd). The size of adipocytes was then calculated using Image-Pro Plus v6.0 (Media. Cybernetics Inc., Silver Springs, MD).

For pancreatic tissues, fresh tissues were fixed with 4% paraformaldehyde for 48 h, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Subsequently, a panoramic scan of the sections was conducted using 3DHISTECH Pannoramic. The number of islets in each section was counted using Case Viewer v2.4. In addition, the embedded wax blocks of mouse pancreatic tissue were sliced into sections approximately 4 μm thick and stained for insulin after standard indirect immunofluorescence staining. Finally, Image Pro Plus v6.0 (Media. Cybernetics Inc., Silver Springs, MD) was used to calculate the insulin positive area and the total area in the scanned images of each section, and divided the insulin positive area by the total area to obtain the percentage of insulin positive area.

2.8 Quantification of mRNA expression in epididymal fat, liver and pancreas

The total RNA was extracted using RNA extraction kits (RNeasy Plus Universal Tissue Mini Kit, 73404, Qiagen) according to manufacturer's instruction. The concentration of the total RNA was determined by agarose gel electrophoresis and NanoPhotometer (NP80, IMPLEN). Then the RNA was reverse transcribed into cDNA according to the instructions of the kit (SuperScriptTM III First-Str Synthesis, 18080051, Invitrogen). Then the cDNA was amplified in a 20 μL reaction system to detect the expression levels of related genes (Table 1) by qPCR with the instrument (qTOWER3G, Analytik Jena). The SYBR Green (IQ SYBR Green Supermix, 170-8882AP BIO-RAD) was used as fluorescence chromogenic system and the primer sequences of related genes are as listed in Table 1. The PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 20 s, 56°C for 30 s, and 72°C for 30 s, and plate reads for 5 s. Gene expression levels were determined using the comparative $\Delta\Delta C_T$ method ($2^{-\Delta\Delta CT}$ method).

2.9 Fecal DNA isolation

Fecal DNAs of mice at week 2 (Trial 1) were isolated according to the method described by Goden et al. (19). The concentration of DNA was measured by NanoPhotometer (NP80, IMPLEN). All DNA samples were diluted to a concentration of 10 ng/μL and stored at -20°C.

2.10 Sequencing of 16s rRNA gene V3-V4 region of gut microbiota and data analysis

The sequencing library of 16S rRNA gene V3-V4 region was conducted following the sequencing guidelines of Illumina with improvement as previously described (20). And sequenced on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA). The raw data were analyzed in QIIME2(Quantitative Insights Into Microbial Ecology, Version 2021.4). The main steps are as follows: import the raw sequences, use the "Cutadapt" plug-in to remove the connectors and primers, and DADA2 to prune the sequence (retain the first 269 bp of all forward sequences and the

TABLE 1 Related genes and the primer sequences for qPCR.

Gene	Primer-F	Primer-R
Fatp1	GGAAGAGCCTCCTCAAGTTCT	GGTCCAGGAGCTGCGTGTCA
Cd36	AGATGACGTGGCAAAGAACAG	CCTTGGCTAGATAACGAACTCTG
Acsl1	GGAGGACCTTGGAAGAGTGAA	ATCTGTGGAAGCGATGAATGC
Cpt1a	AGATCAATCGGACCCTAGACAC	CAGCGAGTAGCGCATAGTCA
Dgat2	GTGCACAAGTGGTGCATCA	CAGTGGGACCTGAGCCATC
Atgl	GCCAACGCCACTCACATCTACG	GACAGCCACGGATGGTCTTCAC
Hsl	ACCATCAACCGACCAGGAGTGCTCTT	GCCCGTCTCGTTGCGTTTGTAGTGT
GAPDH	GACCCCTTCATTGACCTCAAC	CGCTCCTGGAAGATGGTGAT
G6pase	CGAGGAAAGAAAAAGCCAAC	CAAGGTAGATCCGGGACAGA
Pck1	TGTCGGAAGAGGACTTTGAGA	CCACATAGGGCGAGTCTGTC
Pdx1	CCTTTCCCGAATGGAACCGA	GGGCCGGGAGATGTATTTGT
Ins1	TTCTACACACCCAAGTCCCG	AAGTTTTATTCATTGCAGAGGGGTG
Glut2	CAGTCACACCAGCATACACAA	TGATACACTTCGTCCAGCAATG
β-actin	AAGACCTCTATGCCAACACAGT	CTGCTTGCTGATCCACATCTG

first 181 bp of reverse sequences). The sequence information and the original abundance file of amplified subsequence variant (ASVs) were obtained after filtering, denoising, de-chimerism and merging. All samples were rarefied to 24000 reads to normalize sequencing depth and calculate the relative abundance, then the phylogenetic tree was constructed using the "FastTree" plug-in based on the sequence information of ASVs. Taxonomic classification of ASVs was performed using the SILVA132 database.

The alpha diversity of the samples was evaluated using the observed ASVs and Shannon index. Principal coordinate analysis (PCoA) based on Bray-Curtis distance was carried out using the relative abundance matrix of ASVs to evaluate the beta diversity. The statistical significance of gut microbiota among different groups was assessed by permutational multivariate analysis of variance test (PERMANOVA) with 9999 permutations.

Redundancy analysis (RDA) was carried out following the method described by Forester et al. (21). The analysis and plotting were carried out using R (v4.1.3). Firstly, the relative abundance matrix of ASVs was standardized using Hellinger transformation, and the actual intake of carbohydrates, fats and proteins in each group of mice served as environmental variables. Subsequently, the environmental variables were transformed by log1p, the "psych" package was used to analyze the environmental factors, excluding those with a variance inflation factor greater than 10. Redundancy analysis of the microbiota structure was then carried out, with carbohydrate intake and protein intake, or fat intake and protein intake, as environmental variables. The Mantel test was used to analyze the degree of explanation of each RDA axis to the variation of the overall microbiota structure and evaluate the significance. The ASVs related to different environmental factors and significantly affecting the overall microbiota structure were selected based on specific criteria: in the RDA triple sequence diagram, the projection value of the angle with the direction of the environmental factor exceeded 0.6, and the distribution on the RDA1 or RDA2 axis was more than 1.96 standard deviations ($p < 0.05$). The relative abundance of ASVs was visualized using the 'pheatmap' package in R, and differences in the relative abundance of each ASV across different groups were analyzed using the Mann-Whitney U test in GrapadPrism v8.0.

Based on the sequence information of ASVs, the functional genes of the representative strains of ASVs were predicted by PiCRUSt2 (22). Combined with the ASV relative abundance matrix, the relative abundance of functional genes and metabolic pathways in the samples was obtained and annotated based on the KEGG database. Subsequently, the data were visualized using an online platform developed by Chen Tong et al. (23).

2.11 Short–chain fatty acids profiling

To determine the SCFAs in cecum content, all operations were conducted on ice to minimize volatilization. For the standards: accurately measure 400 μL of acetic acid, 200 μL of propionic acid, 200 μL of butyric acid, 20 μL of isobutyric acid, 30 μL of isovaleric acid, and 40 μL of valeric acid, then add water to make a total volume of 5 mL. The standard curve was prepared by diluting 2 mL of the masterbatch to 20 mL, and then further diluted in a two-fold gradient. 200 μL of each gradient standard was used for subsequent acidification, extraction and detection. For the cecal contents, phosphate buffer solution (PBS, 0.01 M) was added (w/v = 1:5), fully mixed and homogenized by Tissuelysser II (QIAGEN, Germany). The mixture was then centrifuged at 16,000 g for 15 minutes at 4°C, and the supernatant was collected and transferred to a new 1.5 mL tube, then filtered through a 0.22 μm aseptic membrane to obtain the fecal water. Next, 200 μL of fecal water of each sample was acidified with 100 μL of 50% (v/v) sulfuric acid.

This mixture was then combined with 400 µL of anhydrous ether, followed by 2 min of static incubation on ice for extraction. The supernatant was collected by centrifugation at 12,000 g for 5 minutes at 4°C. Finally, the concentration of short-chain fatty acids (SCFAs) was measured by gas chromatography (Agilent 6890, Agilent Technologies, USA), using a DB-FFAP (0.25 mm × 30 m × 0.25 µm) column and hydrogen flame ionization.

2.12 Bile acids profiling

The concentrations of bile acids in colon content were determined by liquid chromatography-mass spectrometry (LC-MS). The operations were conducted by Suzhou Panomick Biotechnology company, using an EXionLC liquid chromatograph (EXionLC, ABSCIEX) and mass spectrometer (AB6500Plus, ABSCIEX). Approximately 50 mg of colon content of each sample (n=9 for each group) was used for detection.

2.13 Statistical analysis

In the measurement of physiological index and short-chain fatty acid concentrations, samples from all 10 mice in each group were used. For the measurement of bile acid concentrations, samples from 9 mice in each group were used. Statistical analysis and plots were performed using GraphPad Prism (version8.0). Firstly, ROUT (Q = 1%) was used to test each group of data and eliminate outliers. One-way ANOVA and Tukey's multiple comparison test were used to analyze the differences between groups. The Mann-witney U test was used to compare the relative abundance of intestinal microorganisms between groups. P values < 0.05 were considered statistically significant.

3 Results

3.1 Ketogenic diets induced disorders in both glucose and lipid metabolism in SPF mice

To explore the effects of two distinct ketogenic diets on fat and glucose metabolism in mice, SPF C57BL/6J mice were divided into three groups and fed the specific diets: normal chow diet (NC, comprising 65.6% carbohydrate, 16.3% fat, and 18.0% protein), ketogenic diet 1 (KD1, comprising 8.8% carbohydrate, 73.4% fat, and 17.9% protein, a ketogenic diet commonly employed in clinical studies), or ketogenic diet 2 (KD2, comprising 0.4% carbohydrate, 93.2% fat, and 6.4% protein, a ketogenic diet commonly employed in animal trials). Formulas are provided in Supplementary Table S1. After two weeks of feeding, the fasting blood ketone levels in both KD1 and KD2 groups were significantly elevated compared to the NC group (Supplementary Figure S1A), with KD2 mice exhibiting higher blood ketone level than KD1 mice.

Compared to the NC mice, KD1 mice exhibited increased body weight, while KD2 mice decreased significantly throughout the dietary intervention (Figure 1A, Supplementary Figure S1B). Additionally, fat pad weight and adipocyte size significantly increased in the KD1 group compared to the NC group, while there was no significant difference between the KD2 and NC groups (Figures 1B–D). Furthermore, only the KD2 group showed an increase in liver weight and triglyceride content (Figures 1E, F). Both the KD1 and KD2 groups had significantly higher non-alcoholic fatty liver (NAFLD) scores (Figure 1G). KD2 mice also had significantly higher serum ALT levels compared to the NC mice (Supplementary Figures S1C, D). These findings suggest that both KD1 and KD2 induced lipid accumulation in mice, with an increase in fat pad of KD1 mice but a significant increase in liver observed in KD2 mice.

To probe the causes of lipid accumulation induced by these two KDs, we determined the expression levels of several genes associated with fatty acid oxidation, lipid droplet breakdown, and increased fatty acid uptake in the liver and epididymal fat. Interestingly, we found that the fatty acid uptake related gene *Cd36* was significantly upregulated only in the KD2 group compared to the NC group, while both KD1 and KD2 downregulated the fatty acid oxidation related gene *Acsl1* in the liver (Figures 1H, I). Additionally, in the epididymal fat of KD1 mice, we observed significant downregulation of *Acsl1* and the lipid droplet breakdown-related gene *Hsl* (Supplementary Figures S1F, H). In contrast, KD2 mice exhibited upregulation of the fatty acid oxidation-related gene *Cpt1a* compared to the NC mice (Supplementary Figure S1F). These findings suggest that the KD disrupts the balance between fatty acid oxidation and uptake in the liver and epididymal fat of mice, contributing to disorders in lipid metabolism.

We then assessed the impact of these two types of KD on glucose metabolism. We conducted an oral glucose tolerance test (OGTT) after 2 weeks of dietary intervention and found that KD2 mice exhibited lower fasting blood glucose and insulin levels compared to NC mice, while no significant differences were observed between the KD1 and NC groups (Figures 2A, B). Moreover, both KD1 and KD2 mice showed slower glucose clearance compared to NC mice (Figure 2C). Although the number of islets were similar across all three groups (Figure 2D), the insulin positive area was significantly reduced in the KD2 group compared to the NC group, with no notable difference observed between the KD1 and NC groups (Figure 2E). Interestingly, gluconeogenesis related genes *G6pase* and *Pck1* were significantly decreased in the liver of KD2 mice but not in KD1 mice (Figure 2F). Additionally, both KD1 and KD2 groups exhibited significantly decreased expression of pancreas *Ins1*, with *Glut2* downregulated in the KD2 group. However, expression of *Pdx1*, a gene associated with pancreatic development, did not significantly differ among the three groups (Figure 2G). Overall, these findings indicate that both KD1 and KD2 induced glucose intolerance, likely attributable to insulin synthesis.

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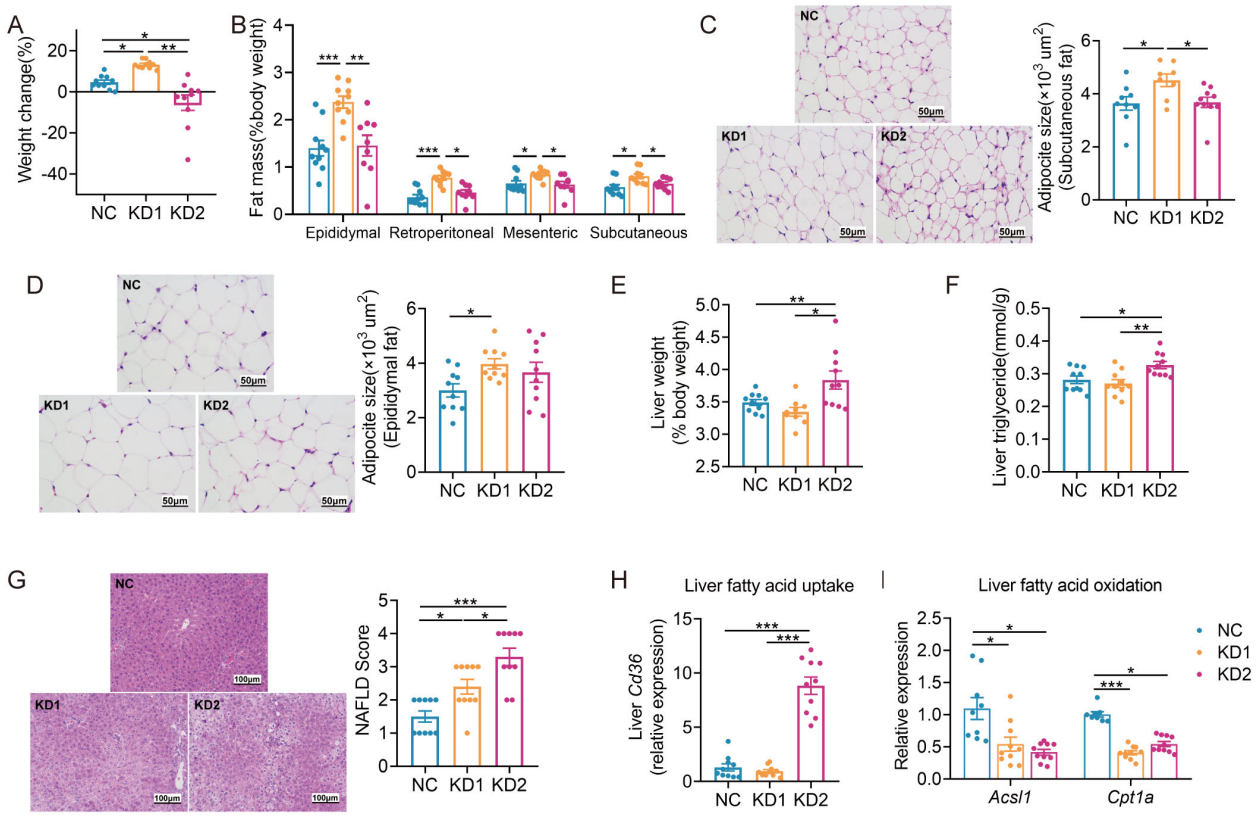

FIGURE 1
KDs induced lipid accumulation in mice. **(A)** Changes of body weight. **(B)** Weight of adipose tissue (epididymal fat, perirenal fat, mesenteric fat and subcutaneous fat, %body weight). **(C, D)** Representative H&E-stained histological sections of subcutaneous fat and epididymal fat (scale: 50 μm) and calculated cell size of adipocytes. **(E)** Liver weight. **(F)** Concentration of liver triglyceride. **(G)** Representative H&E-stained histological sections of liver (scale: 100 μm) and calculated histologic score (NAFLD Score). **(H, I)** Relative mRNA expression of genes involved in **(H)** fatty acid uptake (*Cd36*) and **(I)** oxidation (*Acsl1*, *Cpt1a*) in liver. Data were presented as mean ± SEM and analyzed using one-way ANOVA test, followed by Tukey's multiple comparisons test. ROUT (Q = 1%) was used in each group to eliminate outliers. *p < 0.05, **p < 0.01, ***p < 0.001, n = 10 for each group.

3.2 Ketogenic diets changed gut microbiota structure and bacterial metabolites in mice

Diet is one of the important factors affecting the structure and function of gut microbiota. To explore the effects of the two types of KD on the gut microbiota of mice, we conducted sequencing of the 16S rRNA gene V3-V4 region of fecal samples collected post dietary intervention. The richness and diversity of gut microbiota, as reflected by the numbers of observed amplicon sequence variants (ASVs) and the Shannon index, were significantly decreased in KD1 and KD2 mice compared to the NC mice (Figure 3A). Principal-coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance showed that there were significant differences in microbiota structure among the three groups (Figure 3B). In short, both KD1 and KD2 change the gut microbiota structure of mice.

To explore how gut microbiota contributes to glucose and lipid metabolism disorders induced by ketogenic diets (KDs), we used redundancy analysis (RDA) to identify specific bacterial members responsive to these dietary types. We selected 58 ASVs associated with carbohydrate and fat intake, and 22 ASVs linked to protein intake, based on nutrient consumption in each group (Supplementary Table S2, Supplementary Figure S2). Among the ASVs related to carbohydrate and fat intake, 50 ASVs showed significant correlations with parameters of glucose intolerance (Figure 3C). Notably, three ASVs from the Lachnospiraceae family (ASV124, ASV127, ASV152) were notably enriched, positively correlating with OGTT AUC and negatively correlating with *Ins1* mRNA expression (Figure 3C). Regarding lipid metabolism, a few ASVs correlated significantly with changes in body weight, liver weight, and mesenteric fat, while nearly all 58 ASVs showed positive correlations with liver fatty acid oxidation gene expression (Figure 3C). Furthermore, 55 ASVs were significantly downregulated by both KD1 and KD2 interventions. As for the 22 ASVs related to protein intake, KD2 significantly altered the abundance of all these ASVs (Figure 3D). Among them, 18 ASVs correlated with fasting blood glucose levels and 21 ASVs correlated with changes in body weight (Figure 3D). Additionally, Erysipelotrichaceae (ASV17, ASV1, ASV4) increased significantly in KD1 mice but decreased in KD2 mice, correlating positively with fasting glucose and insulin levels. In summary, the carbohydrate/fat and protein composition of KDs influenced specific gut bacteria in mice, which in turn correlated with parameters of glucose and lipid metabolism disorders.

Short-chain fatty acids (SCFAs) are the products of dietary fiber fermentation by gut microbiota, influenced by diet and the

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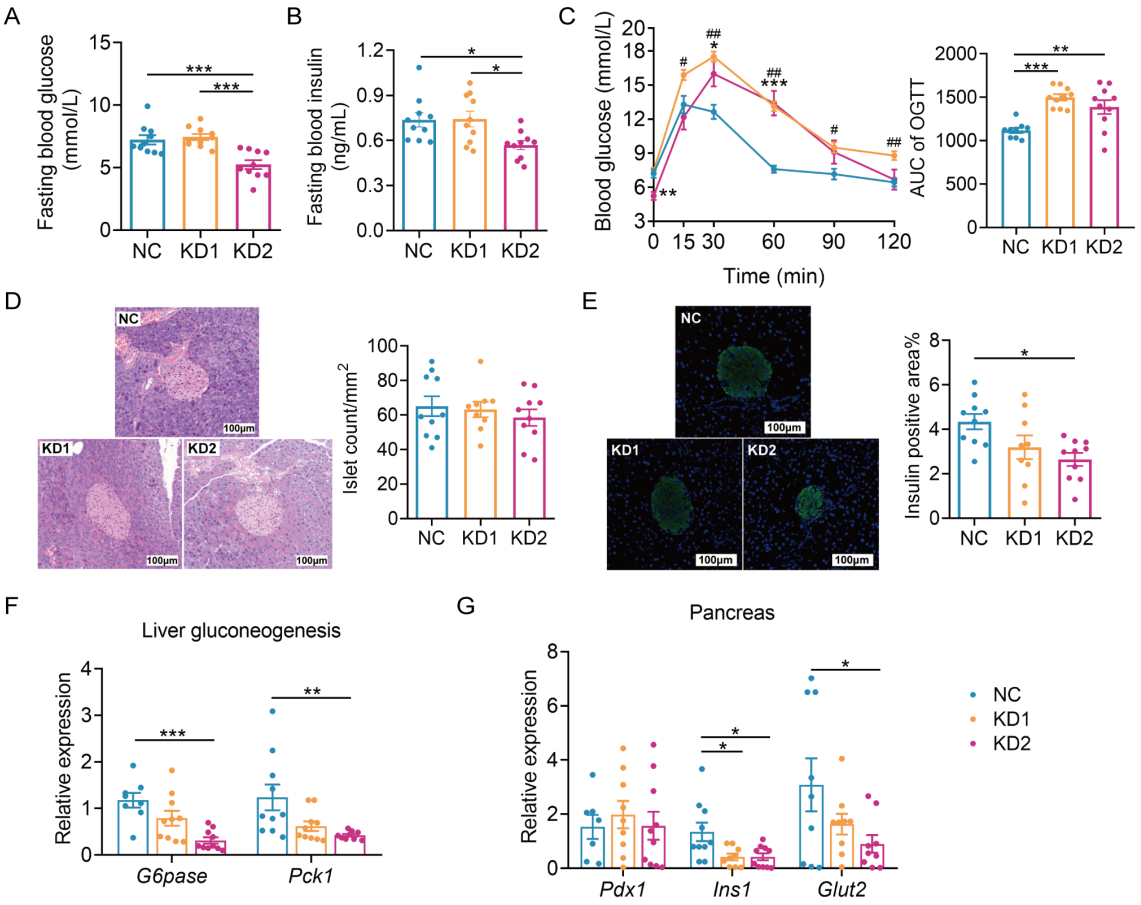

FIGURE 2
KDs induced glucose intolerance in mice. **(A)** Fasting blood glucose. **(B)** Fasting serum insulin. **(C)**Blood glucose curves and areas under the curve (AUC) during the oral glucose tolerance test (OGTT). **(D)** Representative H&E-stained histological sections of pancreas and calculated islet number. **(E)** Representative insulin immunofluorescence-stained (green) histological sections of pancreas (scale bar = 100 μm) and calculated mean insulin-positive area. **(F)** Relative mRNA expression of genes involved in liver gluconeogenesis. **(G)** Relative mRNA expression of genes of pancreas. All data were presented as mean ± SEM and analyzed using one-way ANOVA test, followed by Tukey's multiple comparisons test among NC, KD1 and KD2 groups. In **(C)**, # and * represent the results of Tukey's multiple comparison test between KD1 group and NC group, KD2 group and NC group respectively. ROUT (Q = 1%) was used in each group to eliminate outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, n = 10 for each group.

composition of gut microbiota, and closely related to the glucose and lipid metabolism of the host (24). The contents of each short-chain fatty acid in KD1 and KD2 mice significantly decreased compared to NC mice, with no significant difference in the content of these SCFAs between the KD1 and KD2 groups (Figure 4A). We conducted Spearman correlation analysis between the levels of each short-chain fatty acids and 58 ASVs selected based on RDA analysis associated with carbohydrate intake. We found that 18 ASVs showed significant positive correlations with at least one short-chain fatty acid (Supplementary Figure S3), with certain bacteria within the genera *Parabacteroides*, *Blautia*, and *Butyricimonas* reported to have the ability to produce SCFAs. Among these 18 ASVs, the relative abundance of 16 ASVs was significantly lower in both KD1 and KD2 groups compared to the NC group, while ASV 25 and ASV 26 demonstrated significantly lower relative abundances exclusively in the KD2 group compared to the NC group. These ASVs, responsive to reduced carbohydrate intake in the KD diet, may have the capacity to produce SCFAs, potentially resulting in decreased SCFA levels in the mouse gut and subsequent disruption of carbohydrate metabolism. Bile acids (BAs), crucial for facilitating the decomposition and absorption of dietary lipids, are influenced by the composition and content of dietary fat (25). The gut microbiota metabolizes primary bile acids into secondary bile acids, altering their composition and concentration, thus affecting host glucose metabolism (26). To explore whether gut microbiota participate in the disorder of glucose metabolism caused by the ketogenic diet by affecting bile acid metabolism, we measured the colonic bile acid profile. In comparison to the NC group, the KD1 group exhibited a significant increase in total bile acid content, whereas the KD2 group showed a significant decrease (Figure 4B). Additionally, KD2 significantly decreased the content of primary BAs, while KD1 significantly increased secondary BAs compared to the NC group (Figures 4C, D). Furthermore, we predicted the abundance of genes related to bile acid metabolism pathway of gut microbiota under two types of ketogenic diets based on PICRUSt2. We found that the relative abundance of *cbh*, encoding cholylglycine hydrolase, was markedly lower in the KD2 group compared to both the NC and KD1 groups. Conversely, the abundance of *hbhA*, encoding 7-α-

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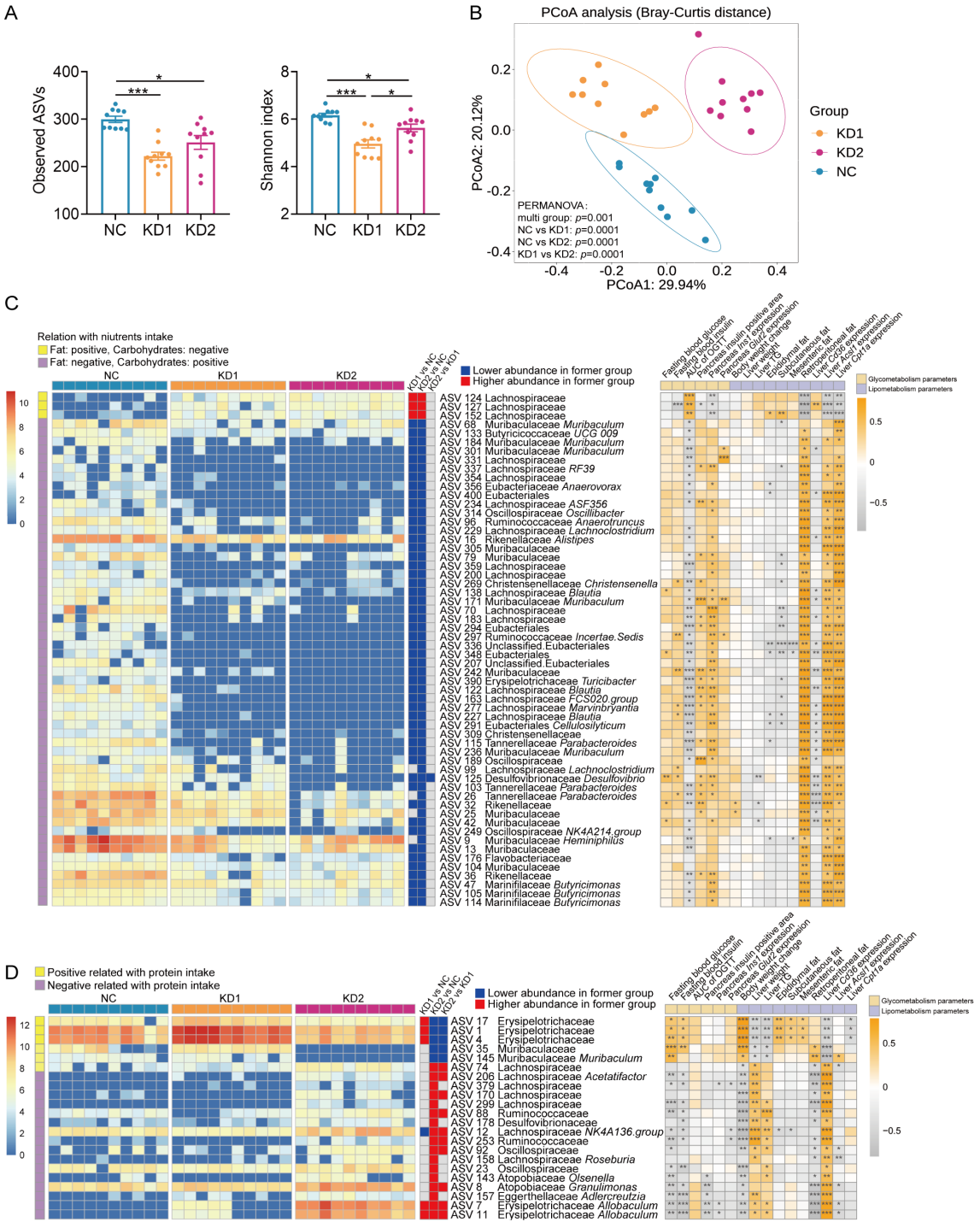

FIGURE 3
KDs changed the structure of gut microbiota. **(A)** Observed ASVs. and Shannon Index. **(B)** Principal coordinate analysis (PCoA) plot of gut microbiota based on Bray-Curtis distance. 95% confidence ellipses were added in different groups, and Permutational Multivariate Analysis of Variance (PERMANOVA) under 9999 permutations was used for microbial structure comparation. **(C, D)** Heatmap of selected ASVs responding to carbohydrates/fat **(C)** and protein **(D)** intake. Left: The heatmap shows the relative abundance (log-transformed) of ASVs in each sample, clustered by ward.D method. Medium: The changing direction of 58 ASVs through pairwise comparison of groups analyzed by the two-tailed tested using Mann-witney test, $p < 0.05$ was considered significant. The blue indicated that the former was significantly lower than the latter, red indicated that the former was significantly higher than the latter and gray indicated that there was no significant difference between the two groups. Right: Spearman correlations between the relative abundance of selected ASVs and the host parameters related to metabolism. Positive correlation (yellow), negative correlation (gray). p values were FDR corrected, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n = 10 for each group.

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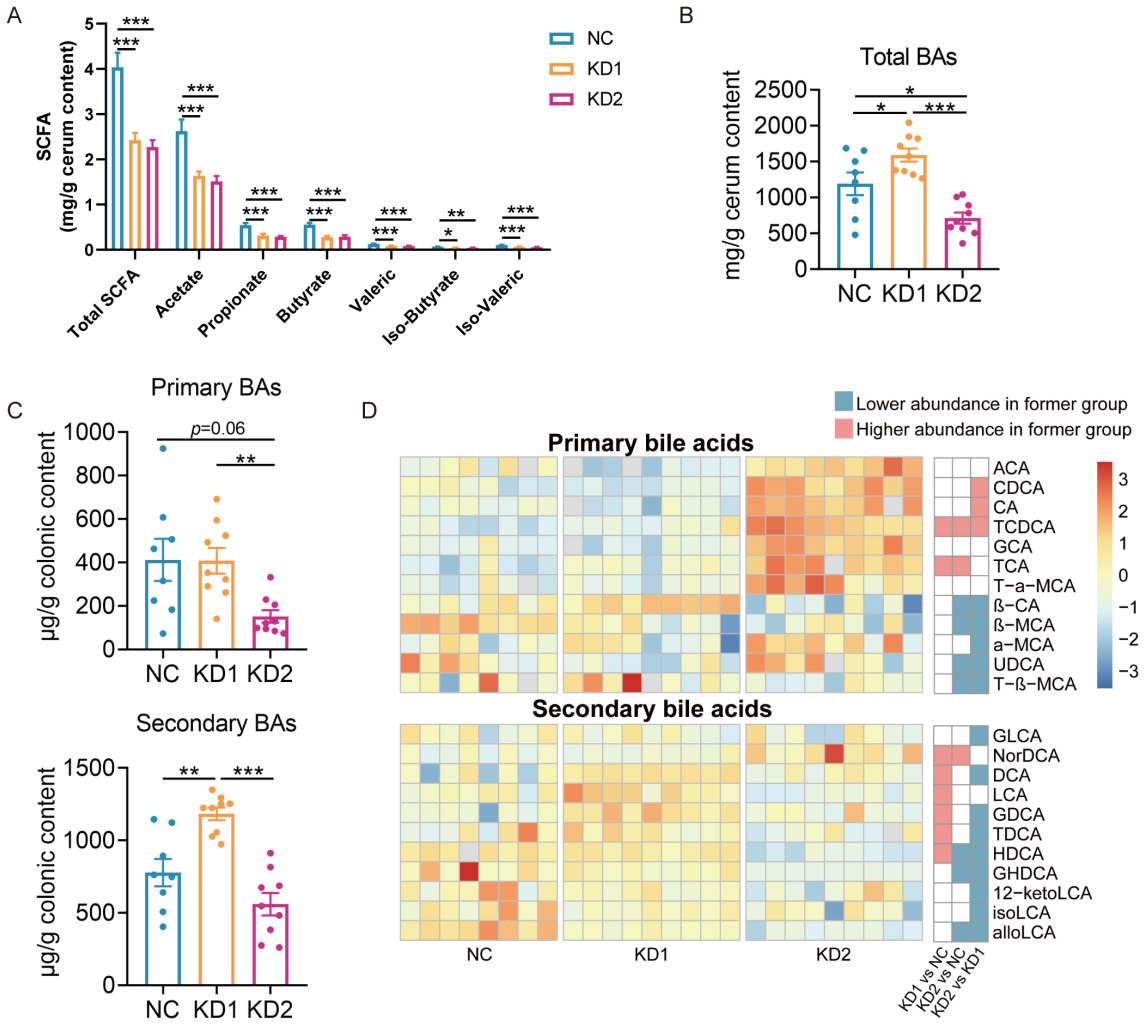

FIGURE 4
Changes of SCFA and bile acids in mice fed with two KDs. **(A)** Concentration of SCFAs (acetate, propionate, butyrate, iso-butyrate, valeric and iso-valeric) in cecal content of mice. **(B)** Concentrations of total bile acids in colon content of mice **(C)** Primary and secondary bile acid content. **(D)** The difference of bile acid content between groups through pairwise comparison of groups analyzed by the two-tailed tested using Mann-witney test, $p < 0.05$ was considered significant, and the blue indicated that the former was significantly lower than the latter, red indicated that the former was significantly higher than the latter and white indicated that there was no significant difference between the two groups. Data were presented as mean ± SEM and analyzed using one-way ANOVA test, followed by Tukey's multiple comparisons test among NC, KD1 and KD2 groups, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. In **(A)**, n = 10 for each group. In **(B, C)**, n = 8 for NC, n = 9 for KD1 and KD2.

hydroxysteroid dehydrogenase, was notably higher in the KD1 group in compared to both the NC and KD2 groups (Supplementary Figure S4). Taken together, these results suggest that KD intervention changes the bacteria related metabolites in the gut.

3.3 KDs induced lipid accumulation in ABX mice, but not glucose intolerance

To find out whether gut microbiota played roles in the mechanisms of KDs induced glucose and lipid metabolism disorders, we treated C57BL/6 mice with a cocktail of antibiotics for 4 weeks, which depleted the gut microbiota by more than 99% (Supplementary Figure S5). Then the mice were divided into three groups and administered their respective diets. After two weeks KD intervention, the blood ketone levels in ABX + KD1 and ABX + KD2 mice increased significantly compared to those in ABX + NC mice (Supplementary Figure S6A).

Similar to conventional mice, the ABX-treated mice had a significant reduction in body weight with KD2 intervention (Figure 5A, Supplementary Figure S6B). Moreover, white adipose tissue weight increased significantly only in the ABX + KD1 group, while liver weight and TG content increased significantly exclusively in the ABX + KD2 group, consistent with conventional mice (Figures 5B–D). Similarly, the area of epididymal and subcutaneous adipocytes increased significantly only in the ABX + KD1 group (Figures 5E, F), while NAFLD Scores were markedly elevated in both the ABX + KD1 and ABX + KD2 groups compared to the ABX + NC group (Figure 5G). Furthermore, the mRNA expression of the fatty acid uptake gene *Cd36* in the liver was also significantly up-regulated in the ABX + KD2 group, while the mRNA expression of fatty acid β-oxidation-

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related genes *Acsl1* and *Cpt1a* was significantly down-regulated in both the ABX + KD1 and ABX + KD2 groups (Figure 5H). Overall, both KD1 and KD2 caused lipid accumulation and fatty acid metabolism disorder under the gut microbiota-depleted condition (Supplementary Figure S6). These findings were consistent with the lipid metabolism phenotype induced by KDs in conventional mice (Figure 1), suggesting that the lipid metabolism abnormalities induced by KD in mice are independent of gut microbiota.

Then we evaluated the effects of the two types of KD on glucose metabolism in ABX mice. Fasting blood glucose and insulin levels were notably elevated in the ABX + KD1 group compared to the ABX + NC group, while no significant difference was observed between the ABX + KD2 group and the ABX + NC group, differing from conventional mice (Figures 6A, B). Moreover, glucose levels at 30 and 60 minutes during an OGTT in the ABX + KD1 group were significantly higher than those in the ABX + NC group after oral glucose administration, but the glucose AUC did not show a statistical difference (Figure 6C). Meanwhile, there was no significant difference in blood glucose levels at each time point or in the 2-hour glucose AUC during the OGTT between the ABX + KD2 group and the ABX + NC group (Figure 6C). The above results showed that the glucose metabolic phenotypes in ABX mice fed with KDs were different from those in conventional mice (Figures 2A–C, 6A–C).

More interestingly, we observed no significant differences in the insulin positive area or the average number of pancreatic islets among the ABX mice fed the three diets (Figures 6D, E). Furthermore, there were no significant differences in the mRNA expression of gluconeogenesis-related gene *G6pase* among the three groups. Only the mRNA expression of *Pck1* was down-regulated in the ABX + KD2 group (Figure 6F), and there were no significant differences in the mRNA expression levels of *Pdx1*, *Ins1*, and *Glut2* —genes related to insulin synthesis and release in the pancreas— among the three groups (Figure 6G). Taken together, these results indicate that ketogenic diets resulted in glucose metabolism disorders in conventional mice but not in microbiota-depleted

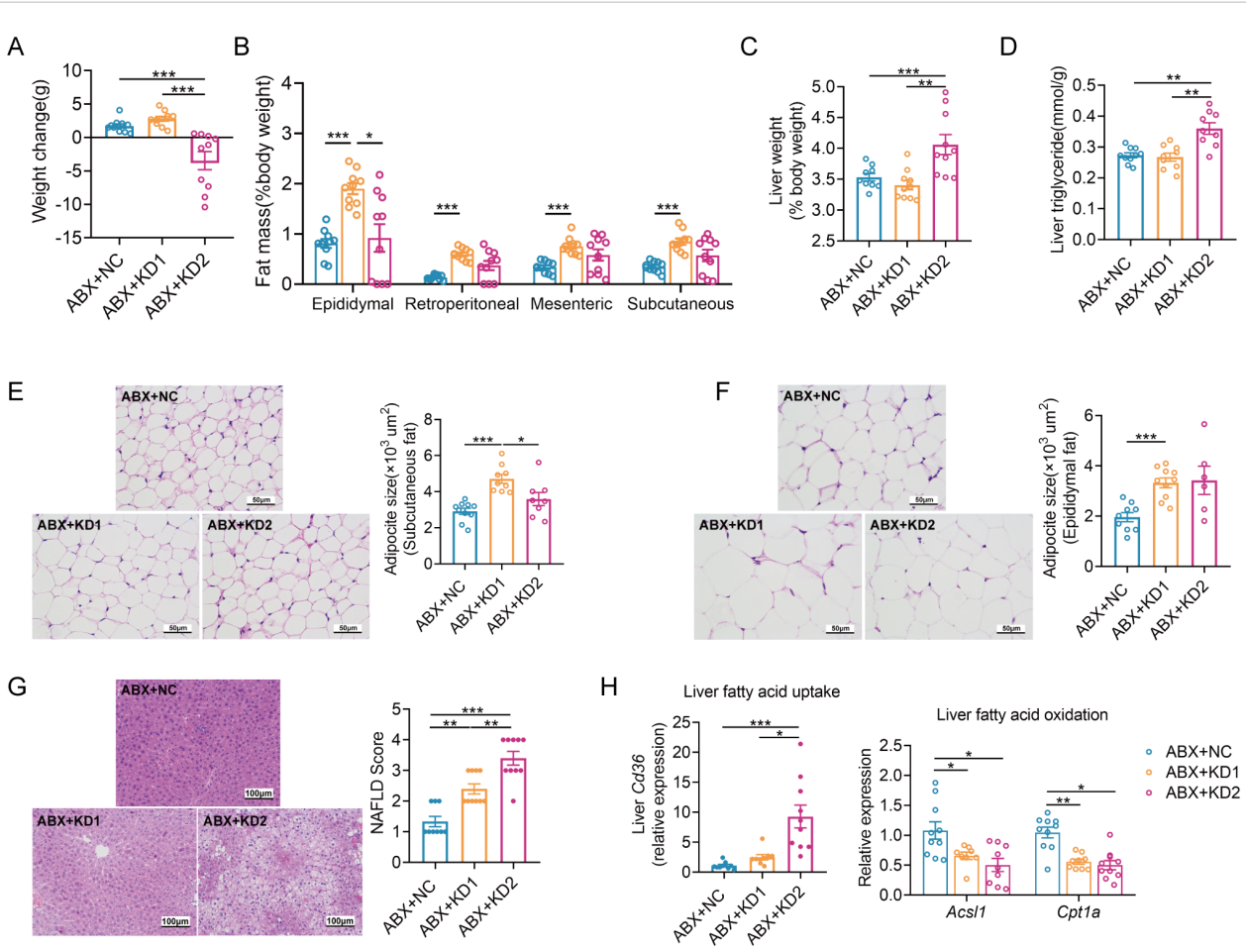

FIGURE 5
Two KDs also induced lipid accumulation in ABX mice. **(A)** Changes of body weight. **(B)**Weight coefficient of adipose tissue (epididymal fat, perirenal fat, mesenteric fat and subcutaneous fat, %body weight). **(C)** Liver weight. **(D)** Concentration of liver TG. **(E, F)** Representative H&E-stained histological sections of subcutaneous fat and epididymal fat (scale: 50 μm) and calculated mean cell area of adipocytes. **(G)** Representative H&E-stained sections of liver (scale: 100 μm) and calculated histologic score (NAFLD Score). **(H)** Relative mRNA expression of genes involved in fatty acid uptake and oxidation in liver. Data were presented as mean ± SEM and analyzed using one-way ANOVA test, followed by Tukey's multiple comparisons test. ROUT (Q = 1%) was used in each group to eliminate outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n = 10 for each group (except **D**). Since the 3 mice in ABX + KD2 group are too thin to collect subcutaneous adipose tissues, the analysis results of 7 mice were shown in **(D)**.

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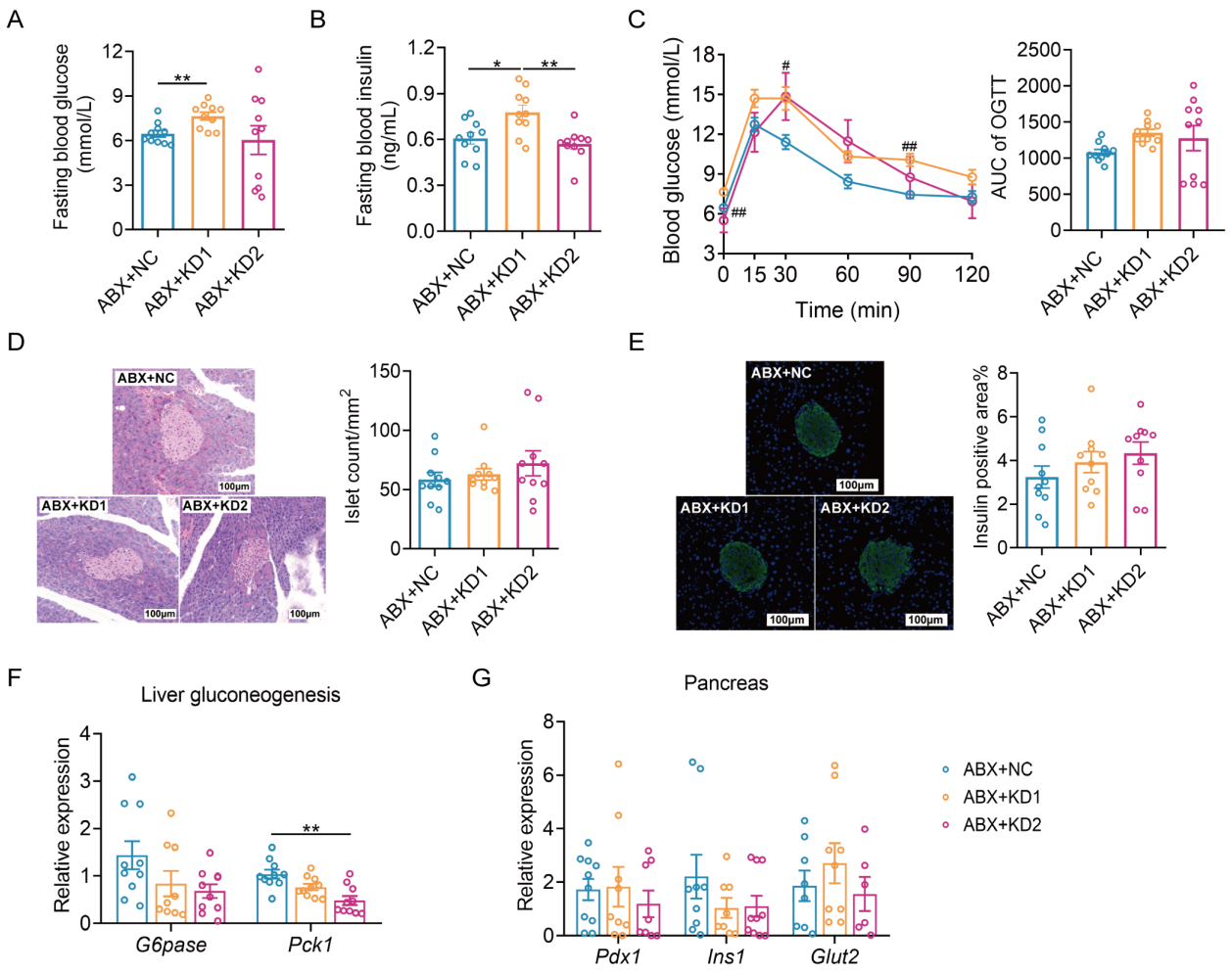

FIGURE 6
Neither KD1 nor KD2 induced glucose intolerance in ABX mice. **(A)** Fasting blood glucose. **(B)** Fasting blood insulin. **(C)**Blood glucose curve and area under the curve (AUC) in the oral glucose tolerance test (OGTT). **(D)** Representative pancreatic tissue H&E staining and calculated islet number and islet area. **(E)** Representative insulin immunofluorescence-stained (green) histological sections of pancreas (scale bar = 100 μm and calculated mean insulin-positive area. **(F)** Relative mRNA expression of genes involved in liver gluconeogenesis. **(G)** Relative mRNA expression of genes of pancreas. All data were presented as mean ± SEM and analyzed using one-way ANOVA test, followed by Tukey's multiple comparisons test among NC, KD1 and KD2 groups. In **(C)**, # and * represent the results of Tukey's multiple comparison test between KD1 group and NC group, KD2 group and NC group respectively. ROUT (Q = 1%) was used in each group to eliminate outliers. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, n = 10 for each group.

mice (Figures 2, 6), suggesting a dependence of ketogenic diet-induced glucose metabolism disorders in mice on gut microbiota.

4 Discussion

Overall, the results of the current work showed that two types of KDs used in clinical studies and animal experiments, caused the disorders of glucose and lipid metabolism in mice. Furthermore, the gut microbiota changed by ketogenic diets involved in glucose intolerance rather than lipid accumulation.

Consistent with previous studies (15, 16), we observed that two types of KDs induced abnormal glucose and lipid metabolism in mice, and both altered the gut microbiota structure. In current research, we found that the gut microbiota mediated glucose metabolism disorders induced by KDs, while KD-induced lipid metabolism disruptions were independent of changes in gut microbiota. Specifically, due to the high fat content in the KD, it affects the expression of genes related to host fatty acid uptake and oxidation, leading to dysregulation of lipid metabolism. Consistently, a previous study has reported that one potential mechanism by which ketogenic diets affect lipid metabolism is by reducing fat synthesis and increasing fat oxidation (27). Interestingly, KD1 resulted in increased peripheral fat rather than hepatic fat accumulation. Concurrently, peripheral fats such as epididymal fat showed increased expression of genes related to triglyceride synthesis, decreased expression of genes related to fatty acid oxidation and lipolysis. Additionally, hepatic fatty acid oxidation decreased. Given the ketogenic diet's inherently high fat content, this difference may be due to imbalances in triglyceride synthesis, fatty acid oxidation, and lipolysis resulting from nutritional imbalances in the diet. To summarize, the ketogenic

diet directly impacts host metabolism, causing abnormalities in host lipid metabolism. In addition, we observed decreased gut microbiota diversity and lower levels of gut SCFAs in KD-fed mice. Furthermore, there was a reduction in the abundance of certain common SCFA-producing bacteria, including *Blautia* (ASV 122, ASV 138, ASV 227) and *Alistipes* (ASV 16) (28). Studies have shown that reduced SCFAs production due to gut microbiota dysbiosis may impair intestinal gluconeogenesis and glucose homeostasis, contributing to glucose metabolism disorders (29). Specifically, SCFAs have been demonstrated to stimulate pancreatic insulin secretion and improve glucose tolerance by acting on G protein-coupled receptors (GPCRs) expressed on pancreatic beta cells (29). It is speculated that the low carbohydrate content in the ketogenic diet leads to decreased levels of SCFAs, which are metabolites produced by the gut microbiota using carbohydrates as substrates, resulting in impaired glucose metabolism in mice. Additionally, both KDs significantly altered the bile acid profile. Research shows that disruption of the gut microbiota can lead to bile acid profile dysregulation, which might contribute to the development of chronic inflammatory diseases such as type 2 diabetes (T2D) and colon cancer (26). In summary, gut microbiota dysbiosis induced by the low-carbohydrate, high-fat ketogenic diet, leading to decreased SCFA levels and alterations in bile acid profile, subsequently resulting in insulin resistance and reduced pancreatic insulin secretion, may represent one of the mechanisms underlying glucose metabolism disorders caused by ketogenic diets.

Previous research suggests that the ketogenic diet effectively reduces fasting blood glucose levels, making it a viable option for managing type 2 diabetes (30, 31). The latest research shows that feeding mice with a ketogenic diet significantly reduces body weight and fasting blood glucose levels (32). In our study, although KD2 lowered fasting blood glucose levels, it still led to impaired glucose tolerance, accompanied by a decrease in insulin secretion. The decrease in the expression levels of *G6pase* and *Pck1* genes indicates a correlation between the decreased in fasting blood glucose and reduced hepatic gluconeogenesis, consistent with the reliance of mice on ketones and fatty acids as energy substrates under the KD. A study indicated that mice with reduced *Pck1* expression develop insulin resistance, hypoglycemia, and hepatic steatosis (33). Therefore, it is hypothesized that the elevated liver triglycerides caused by KD2 may be associated with the high-fat, low-carbohydrate dietary pattern. In summary, the decrease in fasting blood glucose caused by the ketogenic diet does not indicate improved metabolism but rather denotes instability and abnormal glucose processing. Interventions using a ketogenic diet in T2D patients may exacerbate glucose metabolism issues. Furthermore, consistent with the weight loss effects of the ketogenic diet, KD2 resulted in decreased body weight in mice, but this weight loss comes at the expense of hepatic fat accumulation and lipid metabolism imbalance. Prolonged exposure may lead to fatty liver disease and non-alcoholic fatty liver disease. Consistent with our study, previous studies have reported that KD induces glucose intolerance, insulin resistance and hepatic lipid accumulation in mice (16, 17, 34, 35). Recently, research has revealed that just 21 days on a ketogenic diet can induce cellular senescence in various organs such as the heart, kidneys, liver, and brain in mice, highlighting the health risks associated with ketogenic diets (36). In summary, our study underscores the detrimental effects of short-term ketogenic diets on glucose and lipid metabolism. Ketogenic diets should not be considered metabolically healthy, and the associated health risks should be fully considered when using them to treat diseases or facilitate weight loss.

In both research and real life, ketogenic diet formulations vary, which may explain the differing effects observed across studies. The two types of ketogenic diets we employed shared the same nutritional sources but differed in their proportions. Notably, the lower-protein KD2 induced significant hepatic fat accumulation in mice. Previous studies have reported that the KDs with an appropriate amount of protein (caloric content of around 20%) can induce obesity and insulin resistance (16, 37, 38), while those with protein restriction (caloric content of less than 10%) may lead to hepatic lipid accumulation in mice (17, 39, 40). Tricò, D et al. reported that protein restriction regulates fatty acid uptake and metabolism by reducing the expression level of PPARα in the liver (41). Conversely, research has demonstrated that a high-protein diet can promote metabolic health by reducing both liver and overall body fat levels (41). This may explain why an animal KD causes lipid accumulation in the liver due to protein restriction. Additionally, KD does not always lead to weight loss. Studies have shown that the weight loss effect of KD is probably related to ketones, which can reduce appetite and increase energy consumption (42, 43). Fats are predominantly ketogenic, carbohydrates are almost anti-ketogenic, and protein is both ketogenic and antiketogenic. Therefore, only when the ketogenic ratio calculated from the mass of three macronutrients is higher than 1.7 can it cause fat decomposition and weight loss (44, 45). In current research, KD1 caused weight gain and accumulation of subcutaneous and visceral fat. Compared to KD1 mice, the blood ketone level was higher in KD2 mice, indicating a weight-loss effect. Importantly, the two types of ketogenic diets shaped entirely distinct gut microbiota structures, particularly affecting key ASVs responding to protein and fat. Compared to NC diet and KD1, the lower-protein KD2 downregulated the abundance of Muribaculaceae while upregulating taxa such as Lachnospiraceae, Ruminococcaceae, and Desulfovibrionaceae. A study shows that compared to a standard protein diet, a high-protein diet leads to an increase in some disease-associated bacteria (such as *Escherichia/Shigella*, *Enterococcus*, and *Streptococcus*) and a decrease in beneficial bacteria (such as *Ruminococcus*, *Akkermansia*, and *Faecalibacterium prausnitzii*), while a low protein diet leads to a higher abundance of Desulfovibrionaceae (positively correlated with inflammation) (46). Thus, a higher or lower protein intake could have a negative impact on gut microbiota and, subsequently, on health, and there appears to be an optimum protein intake required for normal gut health. In summary, although the ketogenic diet is defined as a diet high in fat, low in carbohydrate, and moderate in protein, in fact, subtle differences in specific nutrients can lead to variations in the effects on host metabolic and its gut microbiota. In future studies, the design of ketogenic diet

formulations needs to be more precise, considering the specific nutrient content and potential synergies between nutrients, as well as their effects on the gut microbiota.

In conclusion, our study reveals that the glucose metabolism abnormalities, rather than lipid metabolism abnormalities, induced by commonly used KDs in clinical studies and animal experiments depend on the gut microbiota, indicating a direction for further elucidating the mechanisms underlying ketogenic diet-induced metabolic disturbances in both glucose and lipid metabolism. It should be noted that a ketogenic diet is a highly unbalanced eating pattern that not only directly affects host metabolism but also indirectly impacts host health through gut microbiota. When utilized for long-term treatment of chronic diseases, it's crucial to carefully design nutrient ratios to mitigate health risks and promote optimal metabolic health for both the host and its microbiota.

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 in the article/Supplementary Material.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WL: Visualization, Writing – original draft, Writing – review & editing, Formal analysis. MG: Investigation, Methodology, Validation, Visualization, Writing – original draft, Formal analysis. ZW: Investigation, Methodology, Writing – original draft. HP: Investigation, Writing – original draft. YL: Investigation, Writing – original draft. CZ: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing, Conceptualization.

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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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1446287/full#supplementary-material

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Gut microbiota and metabolic profiles in adults with unclassified diabetes: a cross-sectional study

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Aims: Our study, employing a multi-omics approach, aimed to delineate the distinct gut microbiota and metabolic characteristics in individuals under 30 with unclassified diabetes, thus shedding light on the underlying pathophysiological mechanisms

Methods: This age- and sex-matched case-control study involved 18 patients with unclassified diabetes, 18 patients with classic type 1 diabetes, 13 patients with type 2 diabetes, and 18 healthy individuals. Metagenomics facilitated the profiling of the gut microbiota, while untargeted liquid chromatography-mass spectrometry was used to quantify the serum lipids and metabolites.

Results: Our findings revealed a unique gut microbiota composition in unclassified diabetes patients, marked by a depletion of *Butyrivibrio proteoclasticus* and *Clostridium* and an increase in *Ruminococcus torques* and *Lachnospiraceae bacterium 8_1_57FAA*. Comparative analysis identified the combined marker panel of five bacterial species, seven serum biomarkers, and three clinical parameters could differentiate patients with UDM from HCs with an AUC of 0.94 (95% CI 0.85–1). Notably, the gut microbiota structure of patients with unclassified diabetes resembled that of type 2 diabetes patients, especially regarding disrupted lipid and branched-chain amino acid metabolism.

Conclusions: Despite sharing certain metabolic features with type 2 diabetes, unclassified diabetes presents unique features. The distinct microbiota and metabolites in unclassified diabetes patients suggest a significant role in modulating glucose, lipid, and amino acid metabolism, potentially influencing disease progression. Further longitudinal studies are essential to explore therapeutic strategies targeting the gut microbiota and metabolites to modify the disease trajectory.

KEYWORDS

diabetes mellitus, gut microbiome, metagenome, metabolites analysis, correlation

1 Introduction

The global incidence of diabetes mellitus has risen dramatically, signifying a major public health dilemma in the twenty-first century (1). Diabetes is characterized by increasing heterogeneity, resulting in a broad spectrum of clinical manifestations and a more diverse range of diabetic subgroups. The increasing incidence of overweight and obesity in individuals with type 1 diabetes mellitus (T1DM) (2), coupled with the occurrence of ketosis or ketoacidosis in types beyond T1DM, further complicates the classification process, especially at the initial diagnosis stage. Consequently, the classification and management of diabetes will become increasingly challenging (3). To underscore this complexity, the World Health Organization (WHO) introduced the category of unclassified diabetes (UDM) in 2019 (4). Nevertheless, the distinctive characteristics and etiological factors of UDM have not been fully elucidated.

Emerging evidence indicates a distinct imbalance in the gut microbiota of patients with childhood-onset T1DM and type 2 diabetes mellitus (T2DM) (5, 6). In childhood-onset T1DM, a reduced *Firmicutes*-to-*Bacteroides* ratio is common, as is an increased prevalence of *Bacteroides* and *Blautia* (7, 8). Research in T1DM animal models suggests that the gut microbiota may influence the autoimmune destruction of pancreatic beta cells by modulating toll-like receptor 2/4 signaling, Th17 cells in the intestinal mucosa, sex hormone levels, and the secretion of pancreatic antibacterial peptides (9–11). Conversely, T2DM patients often exhibit a decrease in butyrate-producing bacteria, notably *Akkermansia muciniphila*, and an increase in bacteria such as *Prevotella copri* and *Bacteroides vulgatus*, which can synthesize branched-chain amino acids (BCAAs), potentially exacerbating insulin resistance (12–14). However, the relationships among the gut microbiota, metabolic profiles, and unclassified diabetes status remain unexplored, emphasizing the necessity for additional research in this population.

In this investigation, we compared the gut microbiota and metabolic profiles among individuals with UDM, T1DM, T2DM, and healthy controls (HCs), elucidating the intricate relationships between the gut microbiota composition, metabolite modules, and clinical phenotypes across these groups. This comprehensive analysis is intended to explore the features of UDM and unravel potential pathogenic mechanisms, contributing to a more nuanced understanding of diabetes subtypes.

2 Methods

2.1 Study participants and recruitment

This cross-sectional study included 18 patients with T1DM, 18 patients with UDM, 13 patients with T2DM, and 18 healthy controls (HCs), all of Han descent and under 30 years. We considered the age of onset ≤ 30 as young-onset. The patients were diagnosed according to the World Health Organization guidelines. T1DM was diagnosed based on the presence of acute ketosis or ketoacidosis, the course of insulin replacement therapy, impaired islet function, or positivity for at least one autoantibody (glutamic acid decarboxylase autoantibodies [GADA], insulinoma-associated antigen-2 autoantibodies [IA-2A], or islet cell antibody [ICA]). T2DM was diagnosed based on a typical history of hyperglycemia, no immediate requirement for insulin treatment, and negativity for islet autoantibodies. UDM was diagnosed through an exclusion-based approach, beginning with genetic tests to rule out monogenic diabetes and assessments to exclude secondary causes like infections or pancreatic disorders. The diagnosis also depended on the patient's clinical profile not matching the criteria for T1DM characterized by autoimmune beta-cell destruction, or T2DM, which involves insulin resistance and relative insulin deficiency. This process ensured the specificity of UDM diagnoses, reserved for cases with unidentified etiologies that do not fit known diabetes types or other defined disorders. All healthy subjects and patients with T2DM tested negative for GADA, IA-2A, and ICA. Additionally, all healthy subjects underwent a standard 75-g oral glucose tolerance test (OGTT) to confirm their normal blood glucose levels. The exclusion criteria for this study included secondary diabetes, acute or chronic inflammatory diseases, infectious diseases, pregnancy, malignant tumors, a history of steroid or immunosuppressive drug use for more than 7 days, a history of treatment with prebiotics, probiotics, antibiotics, or any other medication that could influence the gut microbiota for more than 3 days within the previous 3 months, gastrointestinal diseases, a history of gastrointestinal surgery within the previous year, and hepatic and renal dysfunction. The collected demographic and clinical data included age, sex, diabetes duration, height, weight, body mass index (BMI), systolic blood pressure, and diastolic blood pressure. Additionally, biochemical data such as the 75-g OGTT, C-peptide release test, HbA1c, fasting plasma glucose (FPG), lipid profile, and renal function were collected. All participants provided written informed consent, and this study was approved by the Ruijin Hospital ethics committee.

2.2 Metagenomic analysis of the human gut microbiome

Metagenomic sequencing was utilized to investigate the gut microbiome of the four groups in this study. Fecal samples were collected from each patient for metagenomic analysis. Patients had not been treated with antibiotics for at least one month before sampling. Furthermore, they avoided probiotic-rich foods, including yogurt, for a week before collecting the samples. Each sample was immediately frozen at −80°C or temporarily held in personal freezers at −20°C before being transported to the laboratory within a 24-hour window. Total genomic DNA was extracted using the QIAamp Fast DNA Stool Mini Kit from Qiagen, Germany. Paired-end sequencing was performed on the NovaSeq 6000 platform from Illumina, Inc., in San Diego, CA, USA, at Majorbio BioPharm Technology Co., Ltd., in Shanghai, China. Reads that had adapter sequences or low quality, with a length shorter than 50 bp or a quality value lower than 20, were discarded. The remaining reads were aligned to the *Homo sapiens* genome using the NCBI database(GCA_000001405.28) to remove host

DNA. The short reads were assembled using Megahit. SOAPaligner was used to map high-quality reads with 95% identity to representative genes, and the abundance of genes in each sample was assessed using RPKM. The overall information of all genes in the environment can be summarized by constructing a non-redundant gene set. This involves clustering the gene sequences predicted from all samples using CD-HIT software(http://www.bioinformatics.org/cd-hit/) with default parameters set to 90% identity and 90% coverage. The longest gene in each cluster is selected as the representative sequence to create the non-redundant gene sets, allowing for the exploration of commonalities and differences among the various samples. The results from this step include a table of gene number and length statistics before and after redundancy removal, as well as the base sequences and amino acid sequences of the genes in the non-redundant gene set. For taxonomic annotations, non-redundant gene sets were aligned to the NR database using DIAMOND software (http://ab.inf.uni-tuebingen.de/software/diamond/) with BLASTP (Version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignment parameters were set with an expected value (e-value) of 1e-5 (15), employing the best hit approach.

Using the species annotation results from the taxonomic information database corresponding to the NR library, the abundance of each species was calculated by summing the genes associated with that species. The abundance was then assessed at various taxonomic levels, including Domain, Kingdom, Phylum, Class, Order, Family, Genus, and Species for each sample. To construct the abundance table (abundance profile) at the corresponding taxonomic level. At the domain level, 5 were obtained; at the kingdom level, 13; at the phylum level, 149; at the class level, 262; at the order level, 475; at the family level, 848; at the genus level, 2727; and at the species level, 12836.

We used the KEGG database analysis. For KEGG functional annotation, align non-redundant gene set sequences against the gene database (GENES) using BLASTP (BLAST Version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi), set the expected value e-value for BLAST alignment parameter 1e-5. Functional annotation was performed using KOBAS 2.0 (KEGG Orthology Based Annotation System) based on the alignment results (16). The abundances of this functional category were calculated using the sum of the gene abundances corresponding to KO, Pathway, EC, and Module. There are probably 206112 genes that were obtained and annotated.

2.3 Nontargeted lipidomic analysis

Human serum samples were analyzed using an ultrahigh-performance liquid chromatography high-resolution mass spectrometry/mass spectrometry (UHPLC-HRMS/MS)-based non-targeted lipidomics platform. Lipidomic analysis was performed using a ThermoFisher Ultimate 3000 UHPLC system coupled to a Q Exactive Orbitrap Mass Spectrometry with a Heated Electrospray Ionization Source. The raw UHPLC-HRMS/MS data were processed using Compound Discoverer (version 3.3, Thermo Fisher) with a lipidomics workflow template. This included retention time alignment, compound detection, and compound group and structural identification of lipids using the LipidBlast library (version 68).

2.4 Serum metabolites analysis

The quantification of human serum was performed using the UHPLC-MS/MS platform, which involved several steps, including sample preparation, UHPLC-MS/MS analysis, raw data preprocessing, and the calculation of relative quantification of target metabolites. The metabolites were analyzed using a Thermo Fisher Ultimate 3000 UHPLC system coupled to a Q Exactive Orbitrap Mass Spectrometry in Heated Electrospray Ionization Source with positive and negative modes. The raw data were processed using Xcalibur Software (version 4.0, Thermo Fisher Scientific). In this step, the target metabolites and internal standards were identified, and the integral areas were exported. The relative quantification results were obtained by normalizing the peak areas of the target metabolites to that of the corresponding internal standard.

2.5 Statistical analyses

Differences in clinical parameters were analyzed using the chi-square test or Kruskal–Wallis test, and multiple comparisons were corrected using false discovery rate (FDR) *post hoc* tests. To compare metabolite and lipid profiles, orthogonal projections to latent structures discriminant analysis (OPLS-DA) algorithms were used. Variable importance for the projection (VIP) scores were obtained from the OPLS-DA. A $P_{fdr} < 0.1$ was considered statistically significant for metabolites, and a $P < 0.05$, VIP > 1, and fold change > 1.5 were considered statistically significant for lipids. The Kruskal–Wallis rank-sum test was applied to assess the differences in microbial alpha diversity. Permutational multivariate analysis of variance (PERMANOVA) was used to compare microbiota beta diversity, and redundancy analysis (RDA) was used to evaluate the effects of demographic variables on microbiota community variation. The maximum Pearson correlation coefficient between environmental factors and the sample community can be determined using the bioenv function, which identifies the subset of environmental factors corresponding to the highest correlation. RDA is then performed on the sample species distribution table alongside the environmental factors or their selected subset. The significance of the RDA results is assessed through a permutation test similar to ANOVA, focusing on groups where environmental factors influence differences in microbial communities. Significant differences in the relative abundances of taxa were identified using linear discriminant analysis (LDA) effect size (LEfSe) analysis, and P values were corrected using the Benjamini and Hochberg FDR. Taxa with LDA values > 2.0 and $P < 0.05$ were considered to be differentially abundant, and taxa with $P_{fdr} < 0.1$ were considered to be significantly different (15). LEfSe (http://galaxy.biobakery.org/) is a software tool designed for discovering high-dimensional biological identifiers and revealing

genomic features, including genes, metabolic pathways, and classifications, to distinguish between two or more biological conditions (or taxa). The software first employs the non-parametric factorial Kruskal-Wallis sum-rank test to detect significant differences in abundance, identifying taxa that show marked differences. Next, the Wilcoxon rank-sum test assesses the consistency of these differences across various groups for the identified species. Finally, LEfSe utilizes linear discriminant analysis (LDA) to estimate the impact of the abundance of each component (species, gene, or function) on the observed differential effects.

To determine the effectiveness of the selected features, random forest models were built using microbial features, metabolic features, and a combination of the two types of data to differentiate different groups. We calculated the area under the curve (AUC) for the model using these features, which provided a quantitative measure of the model's ability to distinguish between UNC and HC. The selected features yielded a maximum AUC, indicating strong discriminatory power. The feature set was refined iteratively based on the ROC analysis results, ensuring that only those features that contributed significantly to the AUC were retained. This process helped us focus on the most relevant predictors while minimizing the potential for overfitting (15).These models were built using the randomForest package in R. The data were analyzed using the Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/) (17).

3 Results

3.1 Anthropometric and biochemical measurements of different types of diabetes

The study design is illustrated in Figure 1. In this study, participants with T1DM, T2DM, UDM, and HCs under the age of 30 were recruited following a stringent pathological diagnostic and exclusion methodology. The delineation of unclassified diabetes was based on the World Health Organization (WHO) criteria, excluding known types such as T1DM, T2DM, and hybrid diabetes and specific types such as monogenic diabetes, diseases of the exocrine pancreas, endocrine disorders, drug- or chemical-induced diabetes, infections, uncommon specific forms of immune-mediated diabetes, and other conditions discerned through genetic, clinical, and laboratory evaluations. The biochemical characteristics of the study groups are presented in Table 1. Elevated fasting plasma glucose (FPG) and hemoglobin A1C (HbA1c) levels were observed across all patient groups relative to those of HCs. Compared with

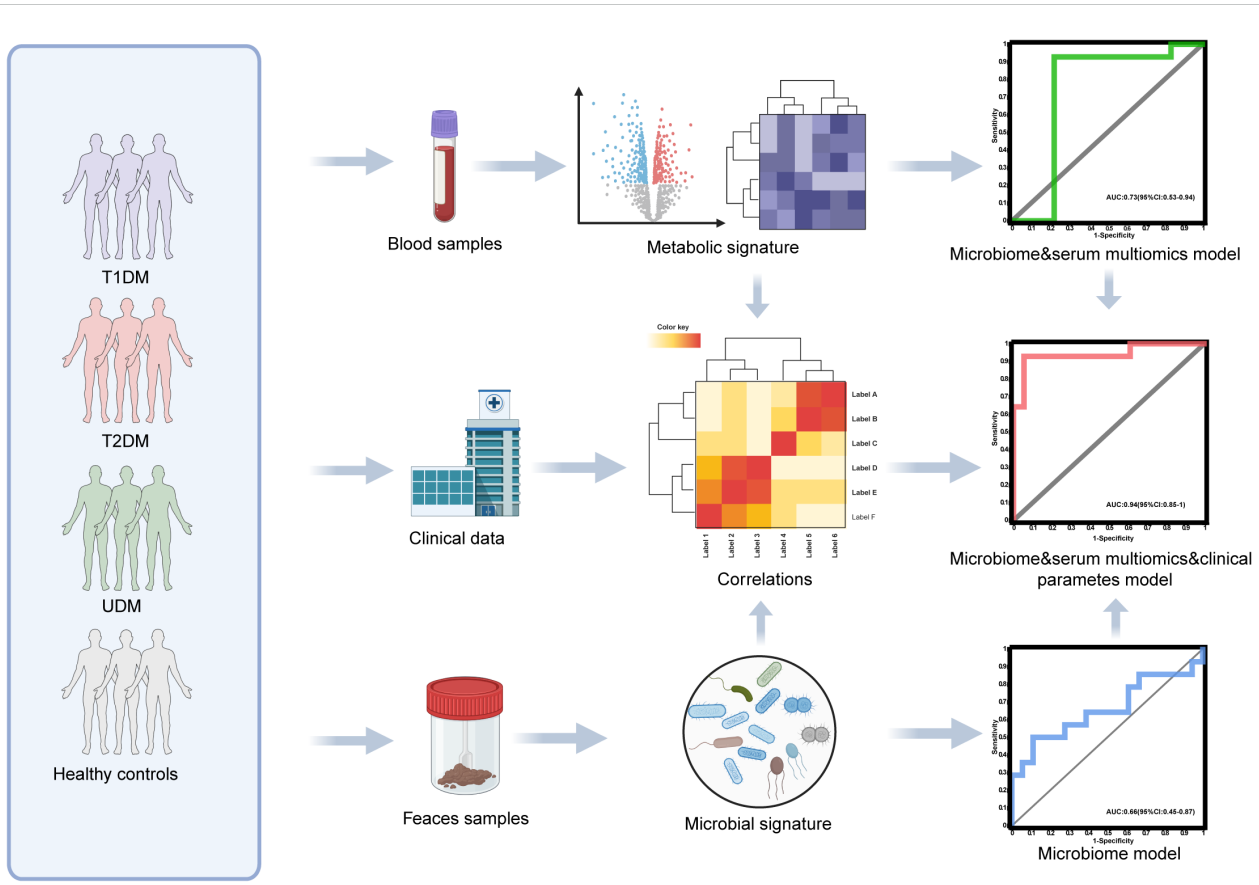

FIGURE 1
Diagram of the study design. Created with BioRender.com.

the T1DM group, the UDM group exhibited increased BMI, FPG, and HbA1c, as well as notably increased uric acid (UA) levels. Furthermore, the homeostatic model assessment for insulin resistance (HOMA-IR) was significantly higher in the UDM group than in the HCs and T1DM groups but remained below the T2DM level. The homeostatic model assessment for beta-cell function (HOMA-β) was significantly higher in the HC group compared to both the UDM and T1DM groups, with the UDM group showing higher levels than the T1DM group. Additionally, levels of LDL, HDL, total cholesterol (TC), and triglycerides (TG) were significantly elevated in the UDM group compared to the HCs group. Furthermore, fasting C-peptide (FCP) and postprandial C-peptide (PCP) levels were significantly higher in the UDM group compared to the T1DM group, but significantly lower than those in the T2DM group.

3.2 Structural modulation of the gut microbiota in the four groups

First, we analyzed the microbial diversity of the four groups. The Chao index results indicated no significant difference in bacterial richness across the groups(healthy controls: 4118 ± 504.9; T1DM patients: 3825 ± 828.7; T2DM patients: 4225 ± 828.1; UDM patients: 3847 ± 688.9; P > 0.05)(Figure 2A). Similarly, the Shannon and Simpson indices also demonstrated no significant differences in bacterial richness among the groups (Supplementary Figure S1). Principal coordinate analysis (PCoA) based on the Bray–Curtis distance revealed significant differences in the overall microbial features across the four groups (PERMANOVA, P = 0.001) (Figure 2B). The bacterial community structure in young-onset UDM patients was significantly distinct from that in HC and T1DM patients (PERMANOVA, T1DM vs HC: P = 0.005; T2DM vs HC: P = 0.005; UDM vs HC: P = 0.001; T1DM vs UDM: P = 0.019), underscoring the different microbial composition associated with UDM.

3.3 Taxonomic changes in microbial composition in young-onset UDM patients

Next, we analyzed the microbial composition at different taxonomic levels, with the phylum and family compositions shown in Figures 2C, D. LEfSe analysis revealed no distinct bacterial phylum structure unique to UDM at the phylum and family levels. *Bacteroidetes* and *Firmicutes* dominated across all groups, followed by *Proteobacteria* and *Actinobacteria*. Significantly, patients with UDM had increased levels of Actinobacteria (0.5636% in HC vs. 3.331% in UDM; P=0.0005314, FDR adjusted=0.03154) and Proteobacteria (3.057% in HC vs. 6.869% in UDM; P=0.001644, FDR-adjusted=0.0515) compared with HC.

TABLE 1 Baseline anthropometric and biochemical variables.

	Healthy controls (n=18)	Unclassified diabetes (n=18)	T1DM (n=18)	T2DM (n=13)	*P* value between all groups
Age (years)	23.00 (21.00-24.00)	22.00 (16.50-27.00)$^{\#}$	22.00 (16.00-26.50)	20.00 (15.00-22.00)	0.963
Age of onset (years)	/	22.00 (15.75-26.25)	21.50 (15.75-26.25)	19.00 (18.00-25.00)	0.931
Male/Female (n)	8/10	10/8	6/12	7/6	0.54
BMI (kg/m^2)	19.85 (19.27-21.55)	26.95 (24.48-30.26)*	21.06 (19.28-25.34)	26.37 (23.38-28.45)*	<0.001
FBG (mmol/L)	4.73 (4.46-4.96)	7.52 (6.19-10.30)*	6.04 (5.49-11.64)*	7.20 (6.94-12.00)*	<0.001
PBG (mmpl/L)	5.85 (4.60-6.81)	15.97 (8.78-18.78)*	16.44 (8.67-25.66)*	14.18 (7.14-17.51)*	<0.001
HbA1c (%)	5.05 (4.67-5.12)	9.65 (7.72-10.47)*^	8.40 (6.70-10.60)*	6.00 (5.20-9.80)*	<0.001
FCP (ng/ml)	1.61 (1.41-2.33)	1.90 (1.68-2.57)$^{\#}$^	0.11 (0.01-0.49)*	3.83 (1.97-3.92)*	<0.001
PCP (ng/ml)	6.92 (5.54-10.89)	4.53 (2.98-5.28)*#^	0.14 (0.01-2.44)*	10.12 (5.98-39.82)	<0.001
TC (mg/dL)	3.90 (3.32-4.38)	4.75 (3.74-5.21)*	4.32 (3.96-4.59)	5.17 (3.67-5.49)	0.054
TG (mg/dL)	0.62 (0.56-0.99)	1.40 (0.80-1.89)*#	0.85 (0.69-1.05)	2.08 (1.29-2.55)	<0.001
HDL (mg/dL)	1.38 (1.30-1.57)	1.12 (0.90-1.46)*#	1.55 (1.23-1.71)	1.02 (0.91-1.07)*	<0.001
LDL (mg/dL)	1.98 (1.68-2.51)	2.66 (1.99-3.75)*	2.52 (2.12-2.90)*	3.19 (2.80-3.56)*	<0.001
UA (μmol/L)	313.00 (255.75-369.00)	362.50 (274.00-445.00)$^{\#}$	286.00 (230.00-345.50)	374.00 (362.00-481.00)	0.025
HOMA-IR	1.33 (0.93-1.65)	2.63 (2.13-5.91)*#^	0.41 (0.11-0.86)*	4.94 (3.10-7.90)*	<0.001
HOMA-β	101.72 (90.18-119.19)	58.19 (29.98-85.25)*#	6.81 (1.46-15.21)*	60.46 (23.88-133.56)	<0.001

The data are presented as the median (25th–75th percentile). *versus healthy controls, P < 0.05; $^{\#}$versus T1DM patients, P < 0.05; ^versus T2DM patients, P < 0.05. T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; BMI, body mass index; FBG, fasting blood glucose; PBG, postprandial blood glucose; HbA1c, hemoglobin A1c; FCP, fasting C-peptide; PCP, postprandial C-peptide; TC, cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; UA, uric acid; HOMA-IR, homeostasis model assessment for insulin resistance; HOMA-β, homeostasis model assessment-β.

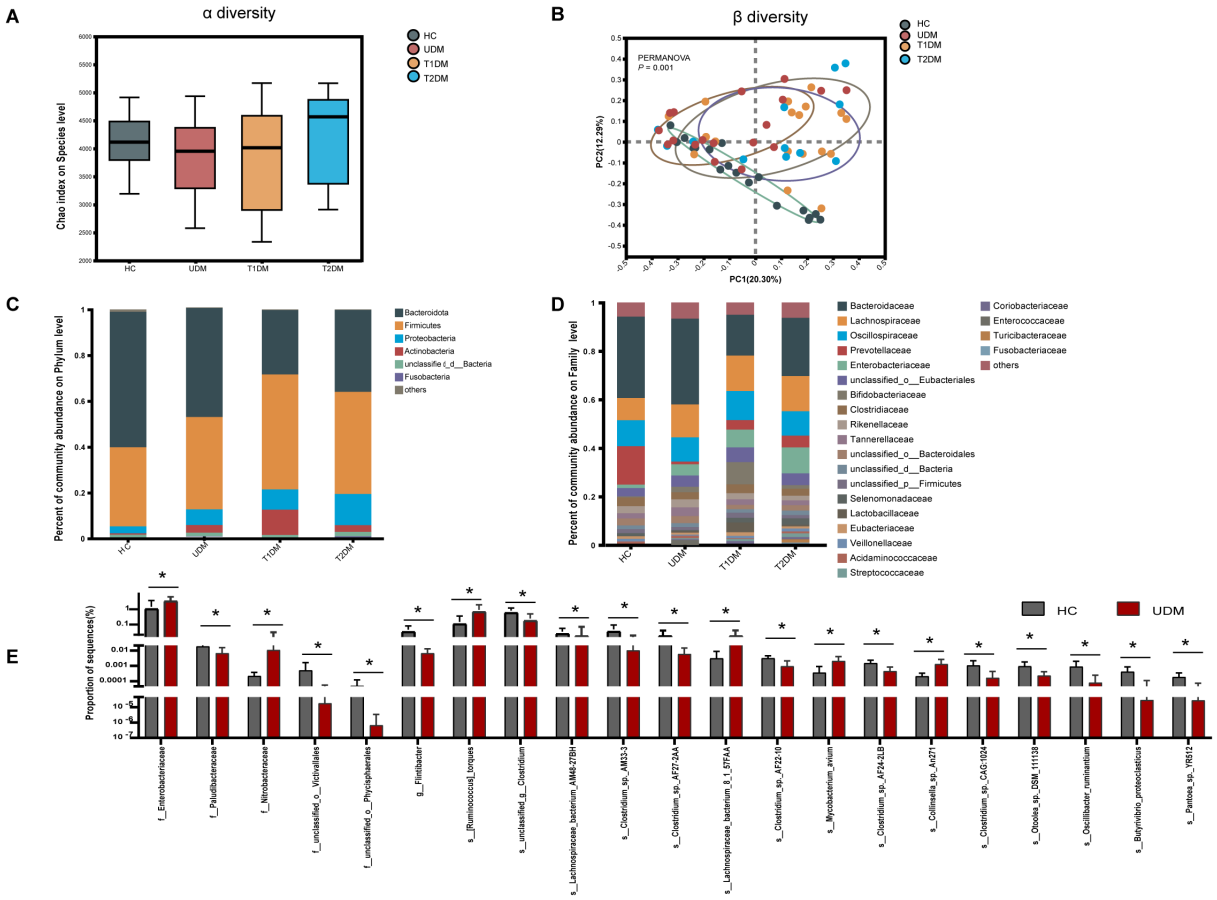

FIGURE 2
The structural shifts and signatures of the gut microbiota in the four groups. **(A)**. Microbial community richness and diversity (Chao 1 index; P = 0.3967). **(B)**. Principal coordinate analysis (PCoA) analysis based on PERMANOVA (P = 0.001). **(C, D)**. The relative abundance of microbial taxa at the phylum and family levels; phyla or genera with a relative abundance <1% in each sample were merged into others. **(E)**. Bar charts showing the relative abundance of taxa that were exclusively altered in patients with UDM compared with HCs. HCs, healthy controls; T1D, type 1 diabetes; T2D, type 2 diabetes; UDM, unclassified diabetes; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance. Bar charts show the mean ± SD. *P_{fdr} < 0.1.(HCs,n=18:UDM,n=18;T1DM,n=18;T2DM,n=13).

LEfSe analysis was employed to discern differentially abundant microbial species among HCs, T1DM patients, T2DM patients, and UDM patients. A total of 81, 34, and 190 species were identified as differentially abundant between T1DM and UDM, T2DM and UDM, and UDM and healthy controls (HCs), respectively(LDA value > 2, P < 0.05) (Supplementary Tables S1-S3). The results from the LEfSe analysis indicate that the UDM group has the fewest differential microbial populations compared to the T2DM group. To determine the potential influence of host factors on microbial composition, we conducted a redundancy analysis (RDA) to ascertain potential confounders within these groups. Key host factors, including age, sex, BMI, and diabetes duration, were integrated into the RDA model. Our analysis revealed that, even after adjusting for these confounding factors, 21 taxa in young-onset UDM patients exhibited significant differential abundance compared to healthy controls (HCs) (LDA value > 2, P_{fdr} < 0.1). Among these, 6 taxa were particularly enriched in UDM patients, including *Lachnospiraceae* and *Enterobacteriaceae*. These taxa are associated with metabolites involved in carbohydrate and amino acid metabolism, suggesting a disturbed gut microbiome's involvement in carbohydrate and amino acid metabolic pathways (18), while there was a notable depletion of 15 species, such as *Flintibacter, Butyrivibrio_proteoclasticus*, *s_Clostridium_sp_AF27_2AA* and *s_Clostridium_sp_AM33_3* (Figure 2E). Additionally, we identified critical functional alterations in the gut microbiota of young-onset UDM patients. There was a significant enrichment of carbon metabolism pathways in these individuals compared to healthy controls, indicating a distinctive metabolic signature. Moreover, the amino sugar and nucleotide sugar metabolism pathways were also significantly enriched in UDM patients compared to those in T1DM and T2DM patients. These findings suggest that the gut microbiota may be involved in the pathogenesis of UDM and shed light on metabolic dysregulation in this disease (Supplementary Figure S2).

3.4 Associations of the microbiota with serum metabolites and lipids

We observed significant differences in serum metabolites between patients with diabetes and HCs (Supplementary Figure S3). Specifically, the numbers of enriched differentially abundant metabolites in the UDM, T1DM, and T2DM groups compared to those in the HC group were 9, 12, and 18, respectively (P_{fdr}<0.1) (Figures 3A-C). Notably, metabolites such as indolelactate, 3-hydroxyisovalerate, acetylcamitine, and 2-hydroxyisocaproate were more abundant in the UDM and T2DM groups than in the HC group. Additionally, we analyzed serum lipids and revealed significant differences across the groups (Supplementary Figure S4), highlighting the metabolic distinctions inherent to diabetes. In patients with UDM, we identified 50 differential lipids, predominantly triglycerides (TGs), which were increased (Figures 4A-C). Subsequent correlation analysis explored the relationships between differentially abundant bacteria and metabolites. This revealed that bacteria enriched in HCs had a strong positive correlation with HC-enriched metabolites but exhibited a negative correlation with diabetes-enriched metabolites (Figure 3D). Notably, a decrease in carnitine and its derivatives, such as valerylcarnitine and lauroylcarnitine, was observed in UDM patients. These compounds are known to enhance glucose utilization, improve lipid profiles, and reduce oxidative stress markers, suggesting that their protective effects may be diminished in UDM (Figure 3D) (19). In individuals with young-onset UDM, an increase in specific metabolic markers, including 3-hydroxybutyric acid, BCAAs, and their catabolic intermediates, was noted. These markers have been associated with an increased risk of transitioning from normoalbuminuria to macroalbuminuria and CKD (20–22). The abundances of bacteria such as *s_Ruminococcus_torques* and *s_Lachnospiraceae_bacterium_8_1_57FAA* were positively correlated with the abundances of metabolites such as 3-hydroxyisovalerate and 3-hydroxybutyric acid, suggesting an increased likelihood of complex diabetic nephropathy in UDM patients. We also observed that amino acids, fatty acid derivatives, and organic acids were enriched in the T2DM group, including alanine, valerylcarnitine, and 2-hydroxyisocaproate, which have been reported to be associated with abnormal fat metabolism and insulin resistance (6, 23, 24).TG and PE were enriched in the UDM and T2DM groups. Additionally, a strong positive correlation between bacteria and lipids (TG and PE) in the UDM and T2DM groups indicates potential parallels in their pathogenic processes (Figure 4D).

3.5 Associations of the altered microbes and metabolites with clinical parameters

To understand the role of the gut microbiota in the progression of diabetes, we analyzed the associations between clinical parameters and differentially abundant bacteria or metabolites in the four groups. We discovered that certain taxa related to young-onset T2DM, including *Streptococcaceae* (25) and *Actinomycetaceae* (26), were significantly

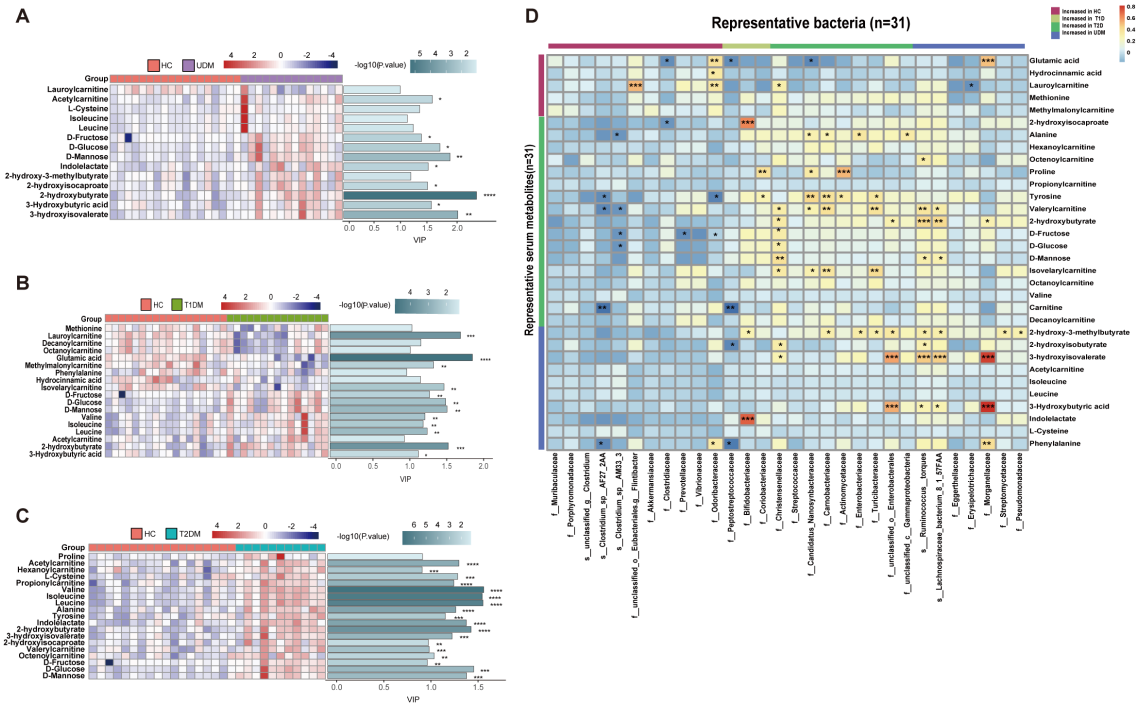

FIGURE 3
Differentially abundant serum metabolites and their associations with gut bacteria. **(A–C)**. Distribution of serum-enriched differentially abundant metabolites between the UDM group and the HC, T1DM, or T2DM group. Variable importance for the projection (VIP) scores were obtained via OPLS-DA. **(D)**. Associations of representative bacteria and serum metabolites that were altered in UDM patients, T1D patients, T2D patients, or both compared with HCs were assessed by Spearman's correlation analysis. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

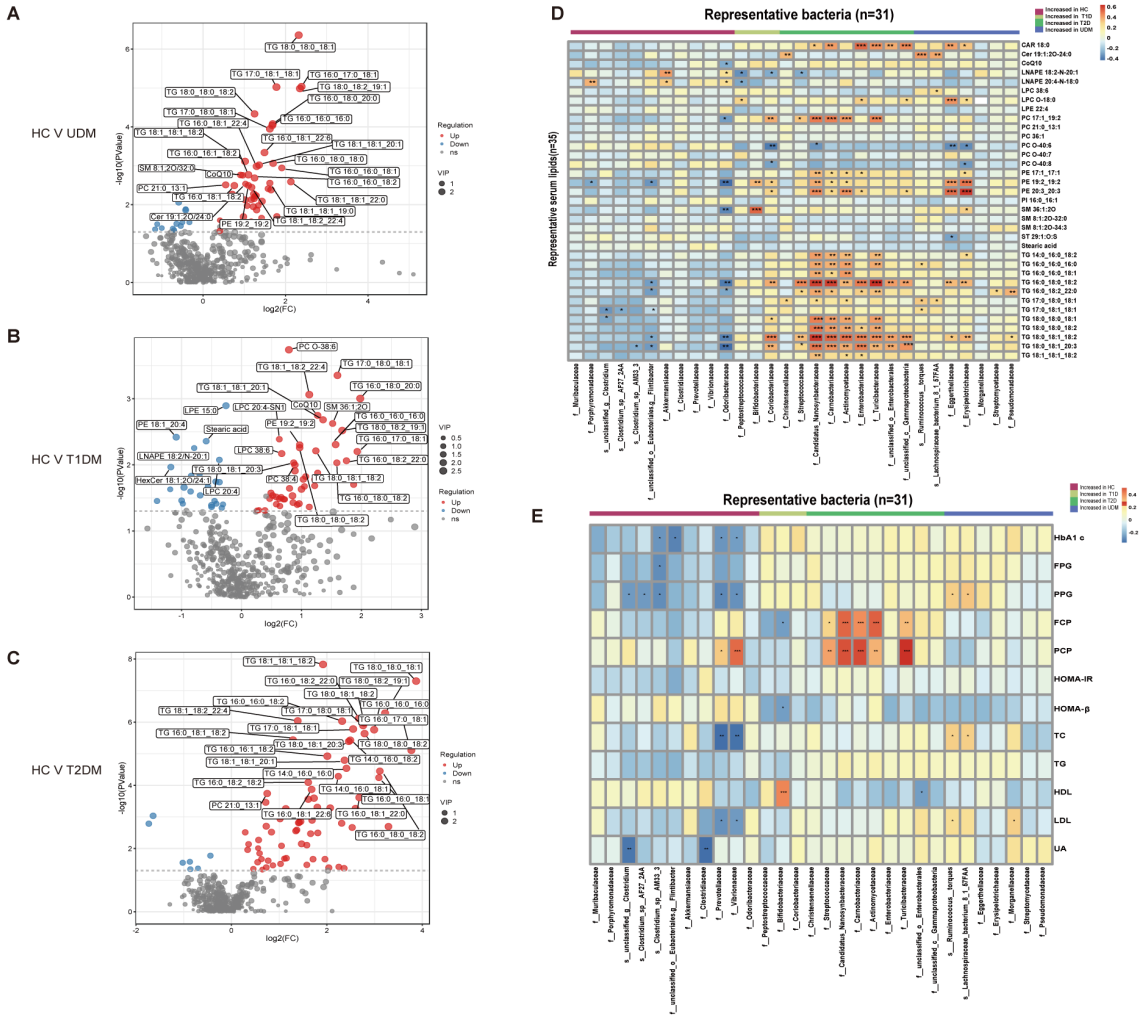

FIGURE 4
Differentially abundant serum lipids and their associations with gut bacteria. **(A–C)** Volcano plots demonstrating differential lipids between HCs and UDM, T1DM, or T2DM patients. p<0.05, VIP>1, and FC>1.5 were used to screen for differentially abundant serum lipids. **(D)** Associations of representative bacteria and serum lipids that were altered in UDM patients, T1D patients, T2D patients, or both compared with HCs were assessed by Spearman's correlation analysis. **(E)** Associations of differentially abundant taxa and clinical parameters in patients. *Pfdr < 0.1, **Pfdr < 0.05, ***Pfdr < 0.01, ****Pfdr < 0.001. FC, fold change; OPLS-DA, orthogonal partial least squares discriminant analysis; VIP, variable influence on projection; HC, healthy controls; T1D, type 1 diabetes; T2D, type 2 diabetes; UDM, unclassified diabetes.

correlated with glucose metabolism and pancreatic beta cell function, corroborating previous findings. These taxa had a positive correlation with PCP and FCP (Figure 4E). In the UDM cohort, bacteria such as *Ruminococcus:torques*, *Lachnospiraceae_bacterium_8_1_57FAA*, and Nitrobacteraceae were positively associated with PBG and FCP. Furthermore, we discovered novel associations between young-onset UDM and increased metabolites such as 2-hydroxy-3-methylbutyrate, 2-hydroxyisobutyraten, and 3-hydroxyisovalerate, which are all positively correlated with PBG, FBG, and FCP. Particularly in UDM, high levels of 3-hydroxyisovalerate, 3-hydroxyisobutyrate, and phenylalanine were strongly related to blood uric acid, indicating their potential role in renal function, suggesting a heightened risk of diabetic nephropathy in UDM patients. In UDM, we observed an enrichment of metabolites integral to amino acid metabolism, including 2-hydroxy-3-methylbutyric acid, cysteine, and phenylalanine. This enrichment aligns with an increase in bacterial pathways for amino sugar metabolites, providing insight into the metabolic landscape of UDM. In T2DM patients, elevated levels of D-fructose, D-glucose, and D-mannose were linked to key glucose and lipid metabolism parameters (Supplementary Figure S5A). Moreover, TG, which was significantly elevated in T2DM patients, correlated strongly with glucose metabolism (Supplementary Figure S5B). Correlations analysis indicates potential links between the gut microbiota, metabolites, and clinical parameters in UDM (Supplementary Figure S6). The results from the LEfSe analysis indicate that the UDM group has the fewest differential microbial populations compared to the T2DM group. A total of 81, 34, and 190 species were differentially abundant between T1DM and UDM, T2DM and UDM, and UDM and HCs, respectively (LDA value > 2, P < 0.05) (Supplementary Tables S1-S3). Meanwhile, the analysis of alterations in gut microbiota functionality revealed that the differential pathways between the UDM and T2DM groups were relatively fewer compared to the other two groups (Supplementary Figure S2). Regarding the differential metabolites compared to the HC group,

the types of differential metabolites in UDM and T2DM were more similar, primarily consisting of TG and amino acid metabolites, and showed a positive correlation with their respective enriched microbial communities (Figures 3D, 4D). Therefore, the gut microbiota structure of patients with unclassified diabetes is relatively similar to that of patients with type 2 diabetes.

3.6 Multiomic classifier discriminating patients with young-onset UDM from patients in the other three groups

To ascertain the potential of the gut microbiota and metabolites as biomarkers for the differential diagnosis of diabetes, we constructed random forest models based on changes in fecal taxonomic or metabolic features between HCs and UDM patients (Supplementary Table S4). The model revealed a bacterial signature of 5 distinct species that could differentiate UDM patients from HCs, with an area under the curve (AUC) of 0.66 (95% CI 0.45–0.87) (Figure 5A). An additional random forest model was assessed for its diagnostic efficacy utilizing a combination of 7 serum biomarkers, including 3 metabolites and 4 lipids. Notably, this model produced an AUC of 0.73 (95% CI 0.53–0.94) for distinguishing patients with young-onset UDM from HCs (Figure 5B). Further enhancement of the model with a panel of five bacterial species, seven serum biomarkers, and three clinical parameters increased its discriminative power, yielding an AUC of 0.94 (95% CI 0.85–1) in differentiating UDM patients from HCs (Figure 5C), demonstrating the potential of this comprehensive approach for accurate diagnosis.

4 Discussion

Eason RJ et al. (27) demonstrated that adults diagnosed with type 1 diabetes who are negative for islet antibodies have genetic and C-peptide characteristics that are intermediate between those of type 1 and type 2 diabetes. This suggests a significant misclassification within this cohort, potentially including individuals with islet antibody-negative autoimmune (type 1) diabetes as well as those with nonautoimmune (predominantly type 2) diabetes who have been erroneously classified. Such misclassification can lead to inappropriate treatment regimens, including unnecessary lifelong insulin therapy, and hinder access to effective type 2 diabetes treatments.

Currently, the high prevalence of type 2 diabetes in adults makes robustly discriminating true type 1 diabetes from atypical presentations of type 2 diabetes challenging. Some reported characteristics of type 1 diabetes in older adults, such as low islet autoantibody prevalence, may reflect the inadvertent study of those with and without autoimmune diabetes, and some research in this area suggests a need to combine clinical diagnosis with gut microbiota and metabolite profile tests in this setting (28–30).

The World Health Organization (WHO) introduced UDM in 2019 when there was no clear diagnostic category (4). In this study, we revealed that unclassified diabetes patients have different gut microbiota and metabolite profiles than healthy individuals as well as classic T1DM and T2DM patients. Remarkably, the gut microbiota of unclassified diabetes patients displayed distinctive characteristics, with significantly increased abundances of *s:_Ruminococcus:torquess* and *Lachnospiraceae_bacterium_8_1_57FAA* and decreased abundances of *s:unclassified_g:Clostridium*, *s:Clostridium_sp:AF27_2AA* and *s:Clostridium_sp:AM33_3* compared with those in the other groups. There was a clear correlation among the gut microbiota, serum metabolites, and clinical phenotypes. Furthermore, the gut bacterial pathway of "Amino sugar and nucleotide sugar metabolism" was significantly enriched in young-onset UDM patients, differentiating them from T1DM and T2DM patients and suggesting that unique metabolic processes are involved in UDM.

In patients with unclassified diabetes, we detected an enrichment of branched-chain amino acids (BCAAs) and their derivatives in the blood, which correlated with glucose and lipid metabolism. Large human population studies have shown that a high intake of dietary BCAAs increases the risk of T2DM (31). In our study, BCAAs and their derivatives might affect glucose metabolism and sensitivity in patients with unclassified diabetes, which was consistent with the functional differences in the bacteria. Metabolites such as indolelactate, 3-hydroxyisovalerate, acetylcarnitine, and 2-hydroxyisocaproate were

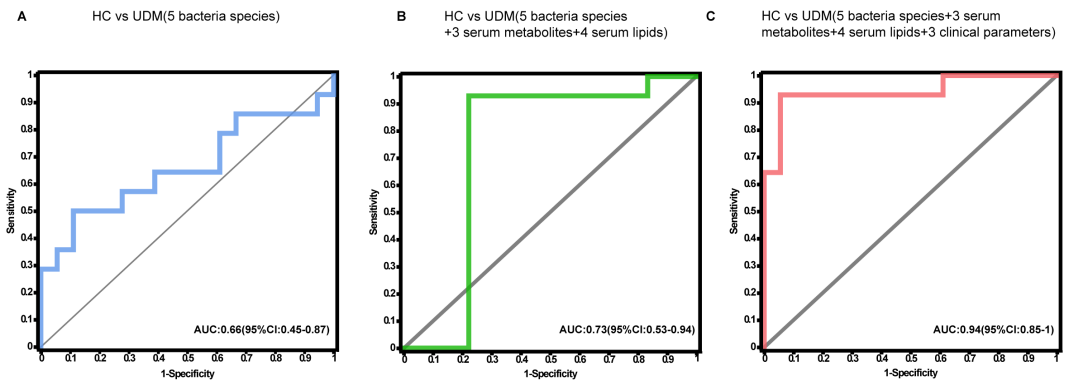

FIGURE 5
Disease classification based on the signatures of the gut microbiome and metabolome. Random forest classifiers composed of bacteria **(A)**, combinations of metabolites **(B)**, and clinical parameters **(C)** were constructed to discriminate patients with UDM from HCs. HC, healthy controls; UDM, unclassified diabetes; AUC, area under the curve.

found at higher concentrations in the UDM and T2DM groups compared to the HC group, and these metabolites are positively associated with the risk of developing T2DM (32–34). In our population, most UDM and T2DM patients exhibited both obesity and deteriorated lipid metabolism, suggesting that these elevated metabolite levels may be linked to the shared obesity characteristics in both groups. Indeed, Patients with UDM or T2DM had We found that, serologically, UDM was more similar to T2DM, but T2DM was dominated by TG enrichment and UDM by amino acid derivatives. Moreover, high levels of 3-hydroxyisovalerate and 3-hydroxyisobutyrate were strongly related to blood uric acid in the UDM group, which could suggest that unclassified diabetes patients had a poor renal function in the subsequent course. Therefore, this finding suggests that patients with unclassified diabetes mellitus need to pay attention to changes in renal function in later follow-up.

Importantly, we developed a prediction model for UDM based on gut microbial signatures and metabolic features, which demonstrated high accuracy in distinguishing patients with this disease from HCs. Furthermore, we have shown that the predictive power of the model can be enhanced by incorporating metabolites, and the utilization of the "5 + 7+3" model enables simultaneous differentiation of patients with UDM from HCs. The differential metabolite composition between the UDM and HC groups was similar to that of the T2DM and HC groups. However, the increasing prevalence of obesity among patients with T1DM due to environmental and lifestyle factors, the presence of ketosis-prone individuals in patients with T2DM and idiopathic T1DM, and the unavailability of autoantibody detection facilities in certain clinics pose challenges in accurately classifying different types of diabetes. In this regard, comprehending the metabolic and microbiota characteristics of unclassified diabetes mellitus patients is crucial for gaining insights into disease pathogenesis and prognosis.

Although our study provides valuable insights into unclassified diabetes, it has several limitations that should be considered. First, the cross-sectional design of our study cannot establish a causal relationship between the identified gut microbiota and young-onset unclassified diabetes. Additionally, the sample size is restricted due to the limited number of adolescent-onset diabetes cases we were able to collect. In future research, we plan to continue gathering data on adolescent cases of type 1, type 2, and unclassified diabetes to develop classification models that can differentiate UNC from other diabetes types based on gut microbiota and metabolites. Moreover, the relatively small sample size and the restriction of subjects to a specific ethnic population and geographic region may limit the generalizability of our results. The taxonomy-based microbiome analysis heavily relies on existing databases for taxonomic assignment in our manuscript. This dependence can introduce biases if the database is incomplete or not representative of the microbial diversity present in the samples.

Finally, despite our efforts to address confounding factors when comparing the three groups (sex- and age-matched patients with comparable demographic characteristics, antibiotic exposure, and comorbidities), our findings could be influenced by other confounders, such as disease duration and dietary intake. Therefore, the significance of these findings should be confirmed through larger prospective follow-up studies involving more diverse ethnic populations and geographic regions.

5 Conclusion

Our study revealed distinct characteristics of the gut microbiota and metabolic profiles in patients with unclassified diabetes, distinguishing them from healthy individuals. Additionally, we observed correlations between these profiles and aspects of glucose metabolism and islet function, suggesting their potential involvement in the development and progression of unclassified diabetes. Importantly, we also found that patients with unclassified diabetes may experience impaired renal function in the future, highlighting the need for careful monitoring. Overall, the findings from this study provide valuable insights that could contribute to the classification and comprehension of diabetes through the identification of novel pathways.

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 in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by Ruijin Hospital Ethics Committee Shanghai JiaoTong University School of Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. The animal studies were approved by Shanghai JiaoTong University School of Medicine Ruijin Hospital Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JZ: Writing – original draft, Writing – review & editing. LW: Writing – original draft, Writing – review & editing. ZZ: Conceptualization, Investigation, Writing – review & editing. DL: Formal analysis, Methodology, Writing – review & editing. RH: Resources, Writing – review & editing. LY: Resources, Writing – review & editing. YZ: Resources, Writing – review & editing. JH: Resources, Writing – review & editing. WG: Funding acquisition, Supervision, Writing – original draft, 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1440984/full#supplementary-material

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The potential mechanism of action of gut flora and bile acids through the TGR5/TRPV1 signaling pathway in diabetic peripheral neuropathic pain

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Diabetic peripheral neuropathic pain (DPNP) is a major complication of diabetes that markedly affects the quality of life and health status of patients. Recent studies have investigated the potential regulatory influence of gut flora and bile acids on DPNP via the TGR5/TRPV1 signaling pathway. Dysbiosis of the gut flora not only directly affects bile acid metabolism but also significantly correlates with diabetes-associated neuropathy through interactions with the bile acid receptor TGR5 and the ion channel TRPV1. This review describes how alterations in the gut flora and bile acid metabolism contribute to the pathogenesis of DPNP through the TGR5/TRPV1 signaling pathway, revealing potential applications for this pathway in DPNP management. Furthermore, experimental and clinical studies have demonstrated the modulation of gut flora and bile acid metabolism as well as targeting the TGR5/TRPV1 signaling pathway as an innovative therapeutic approach. Further studies are warranted to elucidate the underlying mechanism and develop treatment modalities based on gut flora regulation and signaling pathway interventions, thus providing novel insights and approaches for DPNP therapy.

KEYWORDS

diabetic peripheral neuropathic pain, gut flora, bile acids, TGR5, TRPV1

1 Introduction

Diabetic peripheral neuropathic pain (DPNP), which is a common and challenging complication of diabetes mellitus, is mainly caused by diabetes-induced nerve damage and manifests as symmetrical peripheral neuropathic pain in the distal limbs, including mononeuralgia, brachial neuralgia, and lumbosacral neuralgia (1). Current studies have indicated that the prevalence of DPNP among patients with diabetes is as high as 22.8%–31.7% (2, 3). DPNP not only reduces the quality of life of patients but also increases the risk of cardiovascular events and overall mortality (2, 3). The prevalence of DPNP and its impact on patient quality of life are especially evident in patients with type 2 diabetes mellitus (T2DM) (4), with theories in a review supporting the influence of gut flora on DPNP (5). The review outlined the correlation between T2DM and intestinal flora, elucidating its underlying pathological mechanism. Building on this review, the mechanism of action between intestinal flora and bile acid in DPNP through the Takeda G-Protein Receptor 5 (TGR5)/ Transient Receptor Potential Vanilloid Subtype 1 (TRPV1) signal pathway is discussed in detail, to provide theoretical basis for the prevention and treatment of DPNP from the perspective of intestinal flora and bile acid. Diabetic peripheral neuropathy encompasses sensory, motor, and autonomic neuropathy. Implicated causes of peripheral nerve damage include oxidative stress damage; accumulation of sorbitol; advanced glycosylation end products; and a disturbance of hexosamine, protein kinase C, and polymerase pathways. Neurovascular impairment with poor repair processes and endothelial dysfunction have also been implicated (6). However, there are some review papers and retrospective studies that showed the connection between bilirubin serum levels and T2DPNP. The human gut flora is a highly diverse ecosystem comprised of a myriad of bacteria; further, there are theories that metabolites interact with the nervous system in a bidirectional manner through the gut–brain axis (7). Accordingly, gut flora could play different regulatory roles in neurological disorders.

Bile acids, which are synthesized by the liver, are crucial organic molecules that not only play a central role in lipid digestion and absorption but also engage in a complex bidirectional interaction with intestinal microbial communities (8).This interaction affects the diversity and balance of intestinal flora by regulating bile acid metabolism and synthesis (8). Notably, bile acids are involved in numerous physiological processes, including the pathogenesis of diabetic neuropathy, through TGR5 and other receptors (9, 10). In addition, our research group found that the diversity and abundance of intestinal flora in DPNP rats were affected; especially, the relative abundance of Clostridium, Dorea, and Streptococcus increased significantly in the DPNP group. Through a preliminary assessment of serum metabolism in DPNP rats, we found a significant enrichment in the secondary bile acid, deoxycholic acid, within the secondary bile acid biosynthesis pathway. This was significantly upregulated in the serum of the DPNP model group, which suggests a possible relationship between intestinal flora, bile acid, and DPNP. This review article summarizes the potential mechanism underlying DPNP regulation by intestinal flora through bile acid and TGR5 receptors as well as explores the application prospects of this signaling pathway in DPNP therapy. Specifically, this review summarizes the latest research in order to help elucidate the mechanisms underlying the interplay among intestinal flora, bile acids, and DPNP. Furthermore, it explores potential treatment approaches based on this mechanism, which could provide valuable insights for future clinical practice and research endeavors.

2 Intestinal flora, bile acids, and diabetes

2.1 Mechanism of action of intestinal flora

There has been increasing interest in the role of the gut microbiota in the pathogenesis of diabetes. Studies have demonstrated distinct disparities in the gut microbiota composition between individuals with and without diabetes. For example, patients with diabetes have reduced numbers of beneficial gut bacteria, including *Lactobacillus*, *Bifidobacterium*, and *Fecalibacterium prausnitzii*, and an increased number of certain flora potentially associated with diseases, including *Escherichia coli*, *Enterococcus*, and *Clostridium* (11, 12). This shift in microbial composition not only mirrors the diabetes-related alterations in intestinal milieu but may also contribute toward disease onset.

Gut flora has been found to be strongly associated with T2DM (4). The following hypotheses have been proposed (Figure 1):

2.1.1 Bile acid theory

As an important intermediary substance in intestinal and hepatic metabolism, the metabolic process of bile acids is significantly affected by intestinal flora. Disturbances in intestinal flora may lead to reduced production of secondary bile acids, which in turn reduces the activation of bile acid receptors (e.g., TGR5 and FXR) (13, 14), and thus affects glucose metabolism and possibly contributes toward the development of T2DM (15, 16). The activation of bile acid receptors is crucially involved in regulating glucose and lipid metabolism as well as improving insulin sensitivity.

2.1.2 Short-chain fatty acid theory

Intestinal flora can also influence the metabolic state of the host through the production of short-chain fatty acids (e.g., butyric, propionic, and acetic acids). Short-chain fatty acids are an important source of energy; additionally, a reduced number of bacteria that produce short-chain fatty acids results in reduced levels of these fatty acids (17, 18), which impairs pancreatic islet cell function and reduces insulin sensitivity (19) through a decreased ability of the intestines to respond to anti-inflammatory reactions (20); moreover, it results in a weakened ability to activate short-chain fatty acid receptors (21). In patients with diabetes, an abnormal number of short-chain fatty acid-producing bacteria may weaken these protective mechanisms, and thus result in T2DM development.

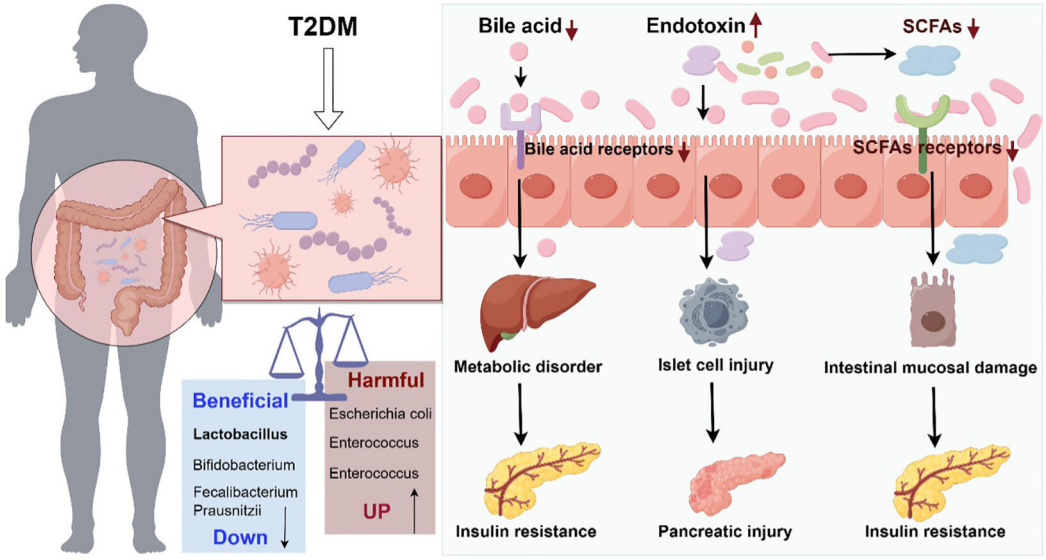

FIGURE 1
Schematic diagram of the role of intestinal flora dysregulation in diabetes (Drawn using Figdraw, color in RGB). The decrease of probiotics and increase of harmful bacteria in the gut tract leads to microecological dysregulation of gut flora. Dysbiosis of gut flora causes abnormal bile acid metabolism and abnormal activation of bile acid receptors, leading to metabolic disorders and insulin resistance. Dysbiosis of gut flora leads to reduced gut barrier function and increased endotoxin penetration, causing islet cell and pancreatic damage. Dysbiosis of gut flora leads to abnormal short-chain fatty acid metabolism, which acts on short-chain fatty acid receptors, causing small gut mucosal damage and insulin resistance.

2.1.3 Endotoxin theory

Dysbiosis of gut flora may result in reduced intestinal barrier function (22) and increased penetration of endotoxins (e.g., lipopolysaccharides), which can enter the circulation and elicit an inflammatory response, leading to insulin resistance (23) and impaired insulin signaling (24), thus contributing to the development of T2DM.

In summary, gut flora significantly influence the host's metabolic state through multiple mechanisms. These include the modulation of bile acid metabolism, generation of short-chain fatty acids, and regulation of endotoxin levels, which collectively influence the development of diabetes.

2.2 Biological role of bile acids

Bile acids can be classified as primary or secondary bile acids based on their origin. Primary bile acids are synthesized directly from cholesterol in hepatocytes, while secondary bile acids are produced through the conversion of primary bile acids by intestinal flora. Specifically, primary bile acids are secreted by hepatocytes and excreted into the intestine via the biliary system, where they are converted into secondary bile acids through the action of intestinal bacteria. Approximately 95% of secondary bile acids are reabsorbed at the end of the small intestine into the hepatic portal vein, where they are transported to the liver for use in a new secretion process, which is a process termed as the enterohepatic cycle of bile acids (25). Bile acids not only help regulate cholesterol homeostasis and promote the digestion and absorption of lipids but also act as signaling molecules involved in metabolic regulation (26). Specifically, bile acids are involved in glucose and lipid metabolism (27), and abnormal glucose metabolism may affect bile acid metabolism in patients with T2DM (28). *In vivo* studies have demonstrated a bidirectional relationship between bile acids and glucose metabolism as well as the involvement of bile acids in T2DM development. Another study found that bile acids can activate the bile acid receptor TGR5, and thus, improve glucose tolerance, insulin sensitivity, and energy metabolism (29). Taken together, these findings demonstrate the crucial role of bile acids in metabolic regulation.

3 Role of the TGR5/TRPV1 signaling pathway in DPNP

3.1 Overview of the TGR5/TRPV1 signaling pathway

TGR5 is a G protein-coupled receptor that binds bile acids and promotes cyclic adenosine 3',5'-monophosphate (cAMP) synthesis via adenylate cyclase, which subsequently activates the protein kinase A pathway and induces the expression of target genes (30). This process induces the production of type II iodothyronine deiodinase, which increases thyroxine synthesis and secretion, thus affecting glucose metabolism. Therefore, TGR5-expressing genes are considered to be closely linked to diabetes mellitus (31). TGR5 is expressed in the nervous system, including in neurons of the dorsal root nerve and in neuroglia (32), which are related to pain-related afferent nerve sensitization. TGR5 activation in neurons of the dorsal root nerve leads to neuronal hyperexcitability and dorsal horn

neurotransmitter release (32). Additionally, bile acids can stimulate TGR5 to induce nociceptor sensitization in the intestinal nervous system, which induces mechanical hypersensitivity (33). For example, TGR5 receptors can play a role in mediating hypersensitivity to bladder distension (30), which further mediates the production of itching sensations. Pruritus can be induced by both histaminergic and non-histaminergic mechanisms, with bile acids being crucial factors in the latter (34). DPNP is characterized by pain resulting from an abnormal somatosensory system, which indicates a potential relationship among bile acids, the TGR5 receptor, and DPNP.

TRPV1 is a cation channel that is highly expressed in dorsal root ganglion neurons; additionally, it is closely associated with DPNP development (35). TRPV1 activation can contribute to the development of DPNP (36); contrastingly, inhibition of TRPV1 channels in the dorsal root ganglion can attenuate DPNP symptoms (37). TRG5 is closely associated with TRPV1 and may act upstream and downstream of the same signaling pathway and jointly participate in the onset and development of DPNP and other related conditions. TRG5 mRNA is exclusively co-expressed with TRPV1 in dorsal root ganglion innervating the bladder (30); additionally, TGR5 activates the cAMP-cAMP response element-binding protein (CREB) signaling pathway. Under cAMP stimulation, CREB activates TRPV1 promoter transcriptional activity, which in turn induces abnormal peripheral pain through activation of the downstream signaling protein kinase C (38). Activated TGR5 modulates itch and provide analgesia by regulating the expression of cation channels such as TRPV1 (39). Further exploration of this pathway would facilitate the elucidation of the mechanisms underlying DPNP and similar conditions as well as the identification of novel treatment targets.

3.2 Relationship between diabetes and TGR5/TRPV1 signaling pathway

TGR5 is expressed in various tissues, and its main function is to maintain blood sugar level and increase energy consumption. Activated TGR5 can up-regulate the production and secretion of glucagon-like peptide -1(GLP-1) in intestinal endocrine cells and improve glucose homeostasis (40). Activation of TGR5 can promote lipolysis and energy consumption (41) and promote metabolic improvement and advanced weight management (42). TGR5 shows a potential anti-diabetic effect, and TGR5 agonists become potential candidates for the treatment of type 2 diabetes, obesity and other metabolic diseases (43).

Type 1 diabetes is an autoimmune disease and TRPV1 is potentially associated with autoimmune abnormalities (44). Impaired muscle Ca^{2+} homeostasis in type 1 diabetic rats was found to be due to TRPV1-mediated attenuation of heat stress tolerance, and capsaicin or other therapeutic strategies that increase Ca^{2+} accumulation via TRPV1 may be more effective than heat therapy in type 1 diabetic patients (45). Continuous oral cilostazol treatment was effective in reducing the level of painful peripheral neuropathy in streptozotocin-induced type I diabetic rats, which may be related to denervation of sensory nerves in the epidermis of the hind paw of DM rats, with a significant reduction in TRPV-1-labeled penetrating nerve fibers (46). Lipid peroxidation products were found to trigger mitochondrial calcium inward flow and mitochondrial dysfunction in endothelial cells in diabetic patients and TRPV1 agonists, and TRPV1 knockout mice were protected from type 1 diabetes-induced endothelial dysfunction and impaired vascular regeneration after arterial injury (47), and TRPV1 activation may be involved in mediating the process of aberrant lipid metabolism that contributes to the onset of diabetes.TRPV1 channel activation plays a protective role in cardiac oxidative/nitrative stress, mitochondrial function, endothelial function, inflammation, and cardiac energy metabolism in diabetic models, and activation of TRPV1 channels can delay the progression of diabetic complications to a certain extent (48). In addition, activation of TRPV1 by capsaicin can mediate insulin signaling-independent glucose oxidation and ATP production in mouse skeletal muscle cells (49), and TRPV1 likewise has potential antidiabetic effects.

3.3 TGR5/TRPV1 cross-pathway affecting DPNP

Other signaling pathways exist that are cross-relational with TGR5/TRPV1 and influence the development of DPNP. It has been found that GPR177 in A-fiber sensory neurons drives diabetic neuropathic pain through WNT-mediated activation of TRPV1, and that GPR177 mediates the secretion of WNT5a from A-fiber DRG neurons into the cerebrospinal fluid (CSF), which is required for the maintenance of DNP (36). Increased P2X3 and TRPV1 activity may mediate the pre-inflammatory spinal cord cytokine release, causing DPNP to occur (50). Additionally α-lipoic acid (ALA) may modulate TRPV1 expression by affecting NF-κB, thereby reducing diabetic neuropathic pain (51). Inosine can attenuate diabetic peripheral neuropathy by modulating GLO1/AGEs/RAGE/NF-κB/Nrf2 and TGF-β/PKC/TRPV1 signaling pathways (52). And ropivacaine can affect peripheral neuropathy in streptozotocin-diabetic rats via TRPV1-CGRP pathway (53). It can be seen that there exists a pathway that crosses with TGR5/TRPV1, which can also affect DPNP development by activating TRPV1.

3.4 Gut flora, bile acids, and the TGR5/TRPV1 signaling pathway

Taken together, patients with diabetes undergo changes in the gut flora composition, which are influenced by environmental factors or their own metabolism. As a result, there are alterations in the intestinal bacterial metabolism of primary bile acids, leading to abnormalities in the metabolism of secondary bile acids, which may, in turn, abnormally activate the cAMP-CREB signaling pathway through action on the TGR5 receptor. This abnormal activation could cause abnormal expression and transcription of TRPV1, leading to downstream signaling that triggers peripheral neuropathic pain. This mechanism describes the entire process from microbial changes to neuropathy in patients with diabetes and provides novel insights into the pathophysiology of DPNP (Figure 2).

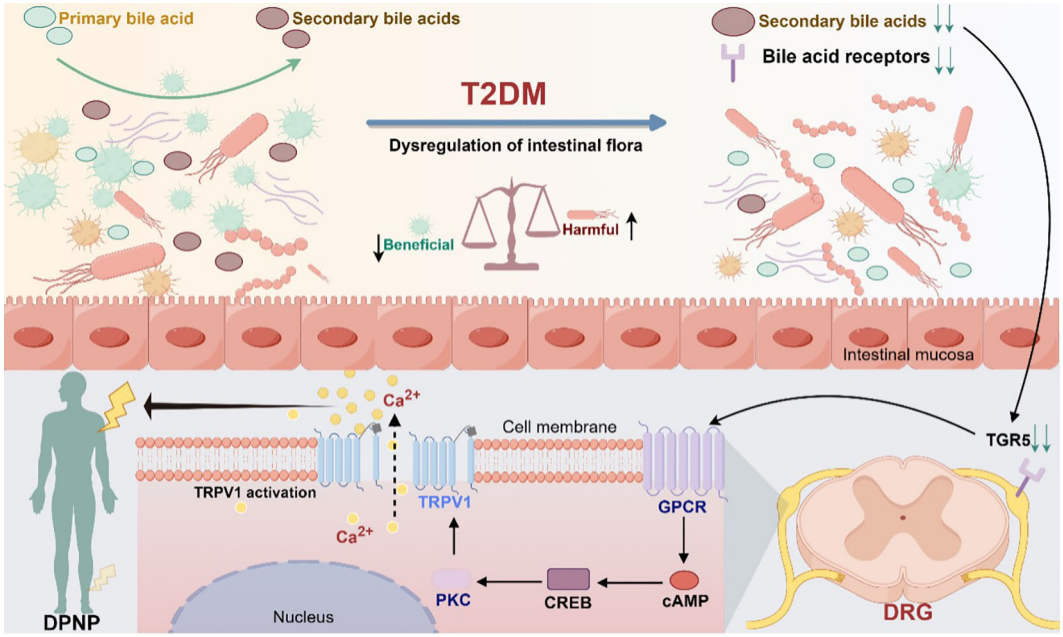

FIGURE 2
Modulation of TGR5 receptors and TRPV1 ion channels in gut microbiota mediating diabetic peripheral neuropathic pain (Drawn using Figdraw, color in RGB). Dysbiosis of gut flora causes abnormal bile acid metabolism and activates the bile acid receptor TGR5, which promotes the synthesis of cAMP through adenylate cyclase. Under the stimulation of cAMP, the cyclic adenosine monophosphate response element-binding protein CREB activates the transcriptional activity of the TRPV1 promoter, which in turn activates the downstream signaling protein kinase C. Through the downstream pathway, it can induce abnormal pain in the peripheral area.

4 Experimental studies and clinical observations

Experimental and clinical studies have provided strong evidence regarding the interaction among gut flora, bile acids, and the TGR5/TRPV1 signaling pathway, as well as their role in the pathogenesis of DPNP. Studies on gut flora have reported that an increase in specific microorganisms such as *Desulfovibrio vulnificus* can increase secondary bile acid production and cecal hydrophobicity, which affects bile acid metabolism and function (54).

In vitro experimental studies have shown that application of TRG5 agonists to bladder-innervating dorsal horn neurons increase intracellular calcium levels, with this process being mediated by TRPV1 channels (55). This suggests a strong link between TGR5 and TRPV1 with respect to neuronal activity and nociceptive transmission. Furthermore, serum lysophosphatidic acid induces limbic activation of heterologously expressed TRPV1, which subsequently regulates neuronal reactivity and sensations such as itching and pain (56). Moreover, mouse studies have demonstrated that TGR5 activation induces itch responses via TRPV1 (32). Taken together, these findings demonstrate that TRG5 and TRPV1 are closely related to neurons; additionally, the TRG5/TRPV1 signaling pathway is crucial for regulating neurosensory sensations, which may be involved in DPNP development.

TGR5 is highly expressed in cells such as monocytes and macrophages, and TGR5 activation produces potent systemic anti-inflammatory properties (57). Dermatitis-lesioned mice have shown TGR5 protein activation and consistently increased TRPV1 expression (58). Furthermore, there is generally reduced expression of inflammatory markers in mice following bilirubin feeding (58). Bile acid synthesis in the liver is correlated with the severity of itching, while bile pigments reduce bile acid levels, decrease the expression of inflammatory markers, and relieve itching (59). Therefore, bile acids may regulate the TRG5/TRPV1 signaling pathway.

Taken together, there is evidence indicating the potential role of the gut flora-bile acid-TGR5/TRPV1 signaling pathway in regulating DPNP development. This study offers new research avenues and identifies potential therapeutic targets for diabetes and its complications. Moreover, these findings underscore the intricate interplay between gut flora, metabolites such as bile acids, and nervous system. They offer a crucial scientific foundation for elucidation of disease mechanisms and development of novel therapeutic approaches.

5 Prevention and treatment of DPNP

The current treatment strategy for the prevention and treatment of DPNP mainly relies on traditional analgesic drugs such as glucose control, amitriptyline, duloxetine, pregabalin, and gabapentin. Some researchers (60) have proposed the idea of combining multiple medications for DPNP treatment; however, not all these medications can completely treat or improve DPNP symptoms. Therefore, it is important to develop novel therapeutic

approaches and targets. We found that intestinal flora, bile acids, bile acid receptor signaling pathways (TGR5 and TRPV1), and DPNP are closely related, and that abnormalities in the composition of intestinal flora in diabetic patients cause abnormalities in bile acid metabolism, which in turn lead to abnormal activation of the relevant bile acid receptor signaling pathways, thus inducing DPNP. Based on this, we believe that regulating the intestinal flora through interventions such as probiotic or prebiotic supplementation, dietary fiber intake, and even fecal transplantation, could correct the intestinal flora disorders in patients with DPNP, which may ameliorate the occurrence and development of DPNP from the source. Currently for regulating the acute effects of this pathway on DPNP is not clear, the role of this pathway on DPNP is more reflected in the chronic effects.

5.1 Regulation of gut flora and bile acids

Dysbiosis of gut flora in patients with diabetes leads to a reduction in probiotics and prebiotics. Accordingly, supplementation with beverages containing probiotics and/or prebiotics can improve T2DM symptoms (61) by restoring the intestinal microenvironment and ameliorating glucose tolerance abnormalities (62). The use of antibiotics to alter the composition of gut flora has been found to alter blood glucose levels and glucose tolerance in animal models (63). Moreover, *Lactobacillus reuteri J1* can alter the composition of gut flora and bile acids, which can be involved in obesity treatment (64). Additionally, dietary fiber supplementation can increase the content of beneficial bacteria in the gut, which in turn establishes functionally active intestinal flora and regulates energy metabolism to a certain extent in order to improve T2DM symptoms (5). In mice, oligofructose supplementation for modulating gut flora has been found to enrich bacteria involved in 6α-hydroxylated bile acid production, which can activate the TGR5-GLP1R axis to improve body weight and metabolism in mice (65). Fecal flora transplantation, i.e., transplantation of gut probiotics from healthy populations to patients, has also demonstrated potential in reshaping the intestinal microecological balance and improving insulin sensitivity (66).Additionally, direct supplementation with bile acids such as ursodeoxycholic acid (67) and glycine ursodeoxycholic acid (68) may facilitate regulation of bile acid metabolism and protection of pancreatic islet β-cells (69); however, its antidiabetic effects remain unclear (70). The regulation of intestinal flora and bile acid metabolism may inform prevention and treatment strategies for T2DM and DPNP (68).

5.2 Regulation of TGR5 receptors and TRPV1 ion channels

Pharmacological modulation of receptors is a valuable approach for developing treatments for various diseases (71). This is because the specificity of the central pathway of sensory input is driven by a combination of receptors and ion channels expressed on sensory endings, which allow differentiation between different stimuli. Specific targeted modulation of the TGR5 receptor on sensory nerve endings is a potential treatment strategy for DPNP. TGR5 agonists have been identified as potential targets for the treatment of T2DM and other metabolic disorders, including obesity, by modulating GLP-1 secretion and increasing energy expenditure in the adipose tissue (43). The downstream TRPV1 channel of TGR5 is involved in the regulation of several important physiological and pathological processes; accordingly, TRPV1 is considered a promising therapeutic target for various diseases, including diabetes mellitus (72). Rat studies have indicated that α-lipoic acid can attenuate neuropathic pain in diabetic rats by downregulating TRPV1 receptors via NF-κB (51) and inhibiting TRPV1 channels (73). Additionally, the antidepressant mirtazapine has shown beneficial effects in diabetes-induced nociceptive hypersensitivity effect, which are achieved by enhancing the inhibitory effect on TRPV1 (74). Taken together, TGR5 and TRPV1 are potential key targets for the treatment of DPNP.

5.3 Combination therapy of gut flora, bile acids and TGR5/TRPV1

Monotherapy directly targeting TGR5/TRPV1 only improves DPNP symptoms and fails to correct the etiology of the disease Not all medications can completely improve symptoms in patients with DPNP (60). Compared with monotherapy, combination therapy involving intestinal flora regulation and bile acids can intervene in the development of diabetes mellitus and DPNP from the source, and the regulation of intestinal flora and bile acids may correct metabolic disorders and improve insulin resistance, which has a better therapeutic effect.

6 Conclusion and outlook

This review discusses the role of the gut flora-bile acid-TGR5/TRPV1 signaling pathway in the development of DPNP. An imbalance in intestinal flora not only affects bile acid metabolism, which, in turn, affects the onset and development of diabetes mellitus, but also is directly related to the development of diabetes-related neuropathy. Novel therapeutic strategies are in development for DPNP by regulating intestinal flora and bile acids as well as targeting TGR5 and TRPV1. We recognize the shortcoming of literature review articles in relation to meta-analysis and systematic reviews. Future studies are warranted to further elucidate the specific mechanisms underlying the interactions among gut flora, bile acids, and the TGR5/TRPV1 signaling pathway, as well as their involvement in DPNP. In addition, it is important to develop novel therapeutic approaches and drugs based on this mechanism, specifically through fecal flora transplantation, probiotic and prebiotic supplementation, and agonists/antagonists targeting TGR5 and TRPV1, for patients with DPNP. However, whether compensatory mechanisms exist in the gut flora or bile acid signaling pathway to attenuate or enhance the effects on the TGR5/

TRPV1 pathway is unclear, and more research is needed for the practical application of this pathway. Accordingly, there is a need for clinical trials to validate the efficacy and safety of these treatment strategies. The ultimate treatment goal is to improve both the symptoms and quality of life of patients with DPNP through modulation of diabetes-related metabolic disorders through these combined treatment strategies.

Author contributions

PC: Writing – original draft, Writing – review & editing, Conceptualization, Formal analysis. XJ: Conceptualization, Writing – review & editing. JF: Conceptualization, Writing – review & editing. CO: Project administration, Resources, Writing – review & editing. YL: Writing – review & editing. JJ: Resources, Supervision, Writing – review & editing. CL: Funding acquisition, Project administration, Validation, Writing – review & editing, Software, Visualization, Conceptualization.

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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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Bile acids as a key target: traditional Chinese medicine for precision management of insulin resistance in type 2 diabetes mellitus through the gut microbiota-bile acids axis

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Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease caused by insulin resistance (IR) and insufficient insulin secretion. Its characteristic pathophysiological processes involve the interaction of multiple mechanisms. In recent years, globally, the prevalence of T2DM has shown a sharp rise due to profound changes in socio-economic structure, the persistent influence of environmental factors, and the complex role of genetic background. It is worth noting that most T2DM patients show significant IR, which further exacerbates the difficulty of disease progression and prevention. In the process of extensively exploring the pathogenesis of T2DM, the dynamic equilibrium of gut microbes and its diverse metabolic activities have increasingly emphasized its central role in the pathophysiological process of T2DM. Bile acids (BAs) metabolism, as a crucial link between gut microbes and the development of T2DM, not only precisely regulates lipid absorption and metabolism but also profoundly influences glucose homeostasis and energy balance through intricate signaling pathways, thus playing a pivotal role in IR progression in T2DM. This review aims to delve into the specific mechanism through which BAs contribute to the development of IR in T2DM, especially emphasizing how gut microbes mediate the metabolic transformation of BAs based on current traditional Chinese medicine research. Ultimately, it seeks to offer new insights into the prevention and treatment of T2DM. Diet, genetics, and the environment intricately sculpt the gut microbiota and BAs metabolism, influencing T2DM-IR. The research has illuminated the significant impact of single herbal medicine,

TCM formulae, and external therapeutic methods such as electroacupuncture on the BAs pool through perturbations in gut microbiota structure. This interaction affects glucose and lipid metabolism as well as insulin sensitivity. Additionally, multiple pathways including BA-FXR-SHP, BA-FXR-FGFR15/19, BA-FXR-NLRP3, BA-TGR5-GLP-1, BAs-TGR5/FXR signaling pathways have been identified through which the BAs pool significantly alter blood glucose levels and improve IR. These findings offer novel approaches for enhancing IR and managing metabolic disorders among patients with T2DM.

KEYWORDS

bile acids, the gut-liver axis, insulin resistance, type 2 diabetes mellitus, traditional Chinese medicine

1 Introduction

The intricate and interdependent relationship between the gut-liver axis and the host has emerged as a prominent area of research in the biomedical field in recent years (1–4). This symbiotic balance exerts a profound influence on human health maintenance and disease onset and progression. Notably, the gut microbiota-bile acids (BAs) axis plays a pivotal role in regulating host immunity, glucose metabolism, and lipid homeostasis, garnering increasing recognition from academia (5–7). The gut microbiota plays a pivotal role in this axis, exerting profound influence on the biological activity of BAs through their transformation and modification (8). Subsequently, BAs function as signaling molecules via nuclear and membrane binding receptors to execute diverse metabolic functions (9). Therefore, the gut microbiota-BAs axis is considered an "endocrine organ" capable of influencing the host's physiological state (10, 11). Recent studies have shown that the gut microbiota-BAs axis also plays a key role in insulin resistance (IR) in type 2 diabetes mellitus (T2DM) (12, 13). The available evidence indicates a significant global increase in the prevalence of diabetes, with IR being prevalent among the majority of diabetic patients (14).

T2DM manifests a diverse array of clinical presentations, primarily due to alterations in pancreatic islet function, IR, metabolic derangements, and other related changes (15, 16). It encompasses a multifaceted etiology, including not only immune dysfunction and genetic predisposition but also crucial lifestyle factors and obesity, which play pivotal roles in its development, consequently resulting in disturbances in carbohydrate, lipid, fluid-electrolyte balance, and protein metabolism (17–20). The prevalence of T2DM is on the rise, indicating a significant inclination towards younger age groups (21). The estimated global prevalence of diabetes among individuals aged 20-79 years in 2021 stood at a concerning rate of 10.5% (22). T2DM, as a chronic condition, exerts detrimental effects on multiple organs, including blood vessels, eyes, kidneys, and even the feet, in the absence of effective management (23). Its uncontrolled state can lead to substantial disability and mortality rates, thereby imposing a heavy burden on healthcare expenditure, with projections indicating that by 2045, the economic cost associated with T2DM will escalate to an astonishing $1,054 billion (22).

Prediabetes, a precarious condition that signifies a heightened risk for the progression to T2DM and serves as a reservoir for the burgeoning diabetic population, has attained a staggering prevalence rate of 50.1% (24). This preclinical condition is fundamentally characterized by IR (25). IR refers to the diminished response of target tissues to the action of insulin, resulting in different levels of impaired glucose and lipid metabolism accompanied by elevated levels of inflammatory mediators (26). Currently, despite the management of prediabetes predominantly revolving around lifestyle modifications, there exists considerable variability in individual responses and adherence. Against this backdrop, Traditional Chinese Medicine (TCM) has gradually emerged as a promising complementary and alternative approach in the prevention and treatment of prediabetes, leveraging its distinct theoretical framework and extensive clinical experience (27–29). By employing a multimodal approach that includes herbal formulas, acupuncture, tuina, and other therapeutic techniques, TCM aims to harmonize yin and yang, unblock meridians,

Abbreviations: AMPK, AMP-activated protein kinase; BAs, bile acids; BSH, bile salt hydrolases; CA, cholic acid; cAMP, Cyclic adenosine monophosphate; CDCA, chenodeoxychilic acid; CYP7A1, cholesterol 7 alpha-hydroxylase; CYP8B1, sterol 12α-hydroxylase; DCA, deoxycholic acid; FGF15/19, fibroblast growth factor 15/19; FGFR4, fibroblast growth factor receptor 4; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide 1; HCA, hyocholic acid; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance; LCA, lithocholic acd; MCA, muricholic acid; NLRP3, NOD-like receptor family pyrin domain-containing 3; NF-κB, nuclear factor kappa-B; PKA, protein kinase A; PPARα, peroxisome proliferator-activated receptor alpha; SHP, small heterodimer partner; TCDCA, taurochenodeoxycholic acid; TCM, traditional chinese medicine; T2DM, type 2 diabetes mellitus; TGR5, takeda G-protein receptor 5; UDCA, ursodeoxycholic acid; VDR, Vitamin D Receptor.

strengthen spleen and kidney functions, thereby enhancing constitutional health and bolstering the innate regulatory mechanisms, ultimately preventing or delaying the onset and progression of T2DM (30, 31).

Therefore, the exploration of novel therapeutic strategies targeting the gut microbiota-BAs axis, particularly those anchored in TCM, holds immense promise for the precision management of IR in T2DM. By delving deeply into the intricate interplay between the gut microbiota, BAs, and IR, our research thus aims to elucidate the mechanisms underlying the efficacy of TCM in modulating this axis and thereby mitigating the progression of T2DM. Understanding the role of the gut microbiota-BAs axis in the pathogenesis of T2DM and the modulation of IR through interventions based on TCM not only advances our knowledge of the complex etiology of the disease but also paves the way for the development of innovative, personalized treatment strategies.

2 BAs

The synthesis and metabolism of BAs constitute a sophisticated and finely tuned process, necessitating the concerted efforts of the liver, intestine, and gut microbiota. BAs can be categorized structurally into free and conjugated forms, while their origins are delineated as primary and secondary BAs. To deepen our exploration of the gut microbiota-BAs axis in T2DM and its modulation by TCM, it becomes essential to elucidate the fundamental mechanisms underlying BAs synthesis and metabolism. This intricate interplay among the liver, gut, and gut microbiota lays the groundwork for comprehending how alterations in BAs composition can impact IR and subsequently drive the progression of T2DM.

2.1 Production and metabolism of BAs

BAs synthesis and metabolism is a complex and finely regulated process involving a synergistic interaction of the liver, gut, and gut microbes (32). BAs are synthesized by hepatocytes utilizing cholesterol as a substrate, starting from two distinct biosynthetic pathways, namely the classical pathway initiated by the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1), and the alternative pathway initiated by sterol 27-hydroxylase (33, 34). In the synthesis of total BAs, the classic pathway predominates, accounting for over 70% of production (35) and contributing approximately 5 g of BAs daily (36). These BAs subsequently conjugate with glycine and taurine to form salts, with a conjugation ratio of approximately 3:1 for glycine to taurine (37). Bile salt export pump (BSEP) serves as the principal BAs efflux transporter, cooperating with multi-drug resistance protein (MDR3) and protein contributing to membrane lipid composition (FIC1) to shuttle BAs from hepatocytes into bile canaliculi (38).

Upon secretion into the gallbladder via the bile duct, BAs are stored awaiting the digestive stimulus of food intake. When food enters the small intestine, the gallbladder contracts, releasing BAs into the duodenum to facilitate the emulsification and absorption (39). Within the gut microbiota environment, primary BAs undergo debinding, dehydroxylation, and epimerization processes to generate secondary BAs such as LCA, deoxycholic acid (DCA), and UDCA (40, 41). Notably, in the realm of secondary BAs, LCA can undergo conversion to UDCA via 7a-hydroxylation, hyodeoxycholic acid through 6a-hydroxylation or murideoxycholic acid by means of 6β-hydroxylation, thereby augmenting the diversity and intricacy of BAs composition (41–43). A portion of BAs is reabsorbed via passive diffusion mechanisms in the small and large intestines, while another fraction undergoes active absorption at the apical membrane of the ileum through the apical sodium-dependent bile acid transporter (ASBT), subsequently entering the portal circulation via the heterodimeric organic solute transporter (OSTα/β) complex (44–46). Approximately 95% of BAs are efficiently reabsorbed in the ileum and transported back to hepatocytes by Sodium/taurocholic acid cotransport polypeptide (NTCP) and organic anion transporting polypeptide (OATP), completing their enterohepatic circulation and metabolism (3, 47). Unabsorbed BAs proceed to the large intestine, with the remainder excreted in feces and urine (48). Under normal physiological conditions, the enterohepatic circulation occurs 8 to 10 times daily (49). In conclusion, the synthesis and metabolism of BAs constitute a highly intricate and precisely regulated system that highlights the harmonious collaboration between the liver, gut, and gut microbiota, ensuring efficient lipid digestion, absorption, and maintenance of metabolic homeostasis. Figure 1 illustrates the process of BAs metabolism.

2.2 BAs and metabolic disorders

BAs, serving as vital signaling molecules between the liver and intestine, play pivotal roles in regulating energy metabolism and maintaining intestinal microecology homeostasis (50). Synthesized in the liver and secreted into the intestine, BAs exert profound influences on the intestinal microbial community structure (51). IR, a hallmark of T2DM, promotes fat accumulation in the liver through multiple pathways, leading to liver damage and subsequently affecting BAs synthesis and secretion (52). BAs are potent antibacterial compounds that play an important role in shaping the ecology of gut microbes (53). The diversity of bacteria in the intestines can tolerate the antibacterial activity of bile salts through a variety of physiological adjustments, and the resistance to bile salts allows certain bile-resistant pathogens to colonize the liver and biliary tract (54). When BAs metabolism is disrupted, resulting in alterations in the gut microbiota composition, increased intestinal permeability, and the entry of more harmful bacteria and endotoxins into the portal vein, further exacerbating liver damage (55). In addition, activation of bile acid receptors triggers mediates the secretion of glucagon-like peptide-1 (GLP-1) by enteroendocrine cells (56). GLP-1 reduces gluconeogenesis, increases energy expenditure, stimulates pancreatic insulin secretion, and attenuates inflammatory responses in immune cells (57, 58). In the context of IR, abnormalities in BAs metabolism may exacerbate liver damage and the risk of metabolic diseases by affecting the intestinal microecology.

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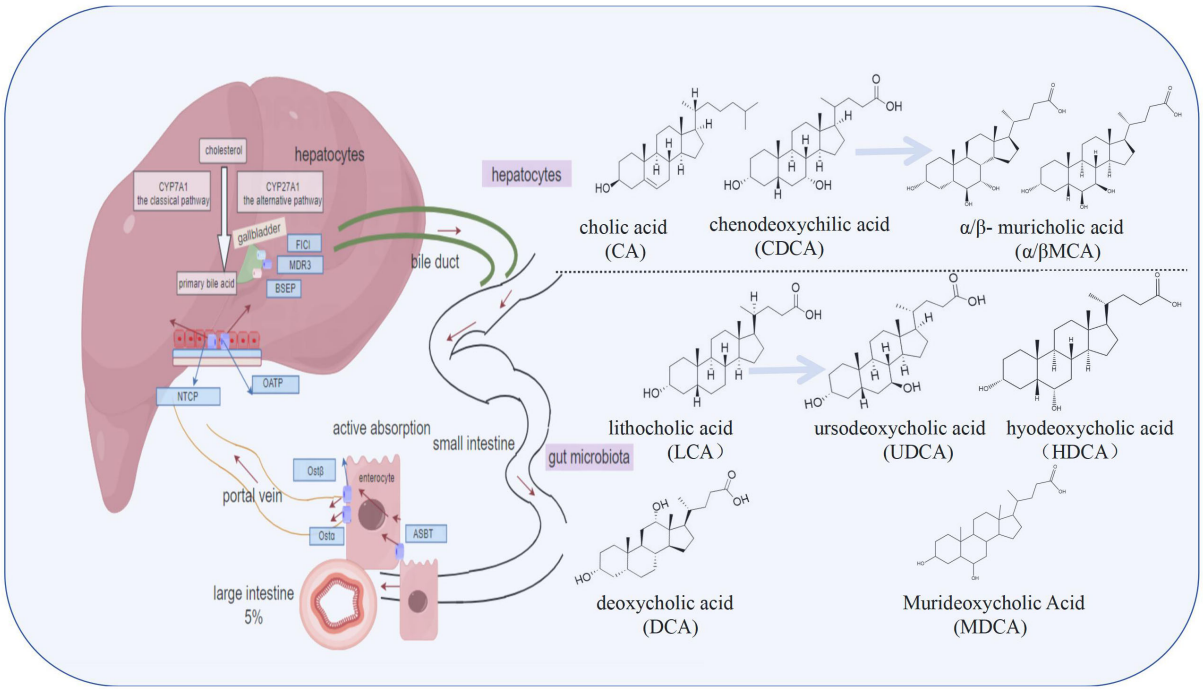

FIGURE 1
The synthesis and metabolic cycle of bile acids (BAs) involve the liver, intestine, and gut microbiota. BAs are synthesized in hepatocytes via the classic pathway and the alternative pathway, and are primarily conjugated with glycine and taurine before being transported to the bile duct by transporters such as bile salt export pump (BSEP). They are stored in the gallbladder and further transformed by gut microbiota into secondary bile acids like lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA), increasing their diversity and complexity. Most bile acids are reabsorbed through passive diffusion or active transport via apical sodium-dependent bile acid transporter (ASBT) in the ileum, entering the portal circulation through the heterodimeric organic solute transporter (OSTα/β), and then transported back to hepatocytes by Sodium/taurocholic acid cotransport polypeptide (NTCP) and organic anion transporting polypeptide (OATP), completing the enterohepatic circulation. Unabsorbed bile acids are excreted in feces and urine.

BAs can be classified into 12α-hydroxylated and non-12α-hydroxylated forms based on their chemical structure, with distinct implications for metabolic health. The main constituents of 12α-OH BAs are cholic acid (CA) and chenodeoxycholic acid (CDCA), and their conjugates with taurine or glycine through amide bonds, while non-12α-hydroxylated BAs include CDCA, Lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) (59, 60). Research has demonstrated that an elevated ratio of 12α-OH BAs to non-12α-OH BAs in serum, which is associated with T2DM, obesity, and IR, may be attributed to an underlying mechanism whereby abnormally elevated glucose and insulin levels induce histone acetylation on the chromatin of CYP7A1, thereby stimulating CYP7A1 synthesis and subsequently leading to an upregulation in the production of 12α-hydroxylated BAs (60–62).

Further observation showed that significant differences were observed in the composition of BAs among the liver, gallbladder, intestine, feces, and serum. There exist notable distinctions between mice and humans, particularly in terms of their BAs composition. Mice exhibit significantly elevated levels of hydrophilic BAs compared to humans (49), which can be primarily attributed to the conversion pathway of CDCA to α/β- muricholic acid (MCA) in mice, leading to a higher proportion of hydrophilic BAs (63). It is worth noting that, while Hyocholic acid (HCA) and its derivatives are present in very low amounts in human blood, they constitute approximately 76% of the total BAs pool in pigs, which have garnered attention due to their remarkable resistance to T2DM (56). These differences not only reflect the species-specific nature of BAs metabolism but also offer new perspectives for studying and understanding the role of BAs in physiology and disease.

BAs is a kind of amphiphilic steroid acid, whose production and diversity are influenced by both host and microbial metabolism (3, 39). Hydrophilic BAs exhibit a certain degree of hepatoprotective effect, whereas hydrophobic BAs possess cytotoxicity, leading to the accumulation of BAs and subsequent damage, necrosis, and apoptosis in liver cells. This disruption can significantly influence gut microbial homeostasis, subsequently affecting overall organismal health. Hydrophobic BAs are considered potential biomarkers for early detection of metabolic disorders. Obese mice demonstrate significantly elevated levels of hydrophobic BAs in both ileal contents and feces compared to normal mice (64). It is noteworthy that hydrophobic BAs possess the ability to inhibit intestinal cholesterol absorption in a high-fat diet context. As the concentration of hydrophobic BAs increases, it consequently imposes a greater metabolic burden on the liver, thereby further escalating the risk of developing metabolic disorders (52). Interestingly, mice with the cytochrome P450 family 2 subfamily c polypeptide 70 knockout display a more hydrophobic composition of BAs pool resembling that of humans (65). Therefore, monitoring and analyzing hydrophobic BAs play a crucial role in the prevention and treatment of metabolic disorders. These findings not only enhance

our comprehension of the disparities in BAs metabolism between rodents and humans but also have significant implications for utilizing mouse models to investigate BAs-related diseases in humans.

3 T2DM-IR and structural changes of gut microbiota

In regulating host metabolism, the gut microbiota plays a pivotal role, particularly as it exerts its influence through intricate and finely tuned interactions that affect multiple facets, including insulin signaling, glucose and lipid metabolism, and protein metabolism, all of which are crucial in the onset and progression of IR (66). Homeostatic model assessment of insulin resistance (HOMA-IR) is an indicator of IR, with its levels directly influenced by the composition of the gut microbiota (67). Specifically, three bacterial taxa, namely Cyanobacteria, Acidobacteria, and Gemmatimonadetes, exhibited a negative correlation with HOMA-IR, suggesting their potential to ameliorate IR (68). Further research also pointed out that effective treatment interventions can reinstate the abundance of microbiota, thereby ameliorating associated symptoms (69–71). It is noteworthy that the improvement of insulin sensitivity by metformin, a widely used hypoglycemic drug, is closely related to the regulation of gut microbiota, demonstrating the complexity of drug-microbial interactions (72). Sun et al. found that metformin can effectively prevent and treat impaired glucose tolerance and IR by inhibiting intestinal the Farnesoid X receptor (FXR) signal through the gut microbiome in a non-AMP-activated protein kinase (AMPK)-dependent manner (73). This finding not only deepens our understanding of the mechanism of action of metformin, but also highlights the importance of gut microbiota in the efficacy of the drug. Furthermore, TCM, with its unique theoretical framework and therapeutic advantages, has demonstrated considerable potential in the treatment of chronic metabolic diseases associated with IR (74, 75). In recent years, the gut microbiota has emerged as a novel bridge linking TCM therapies to disease improvement, becoming a frontier and hot research area in exploring the mechanisms of action of Chinese medicinal herbs (76).

TCM boasts a rich history in the management of T2DM. As research progresses, an increasing body of evidence demonstrates that TCM can effectively ameliorate IR through the regulation of gut microbiota (77–79). The compound salvianolic acid A, derived from Salvia miltiorrhiza and recognized as an effective therapeutic agent for T2DM, not only demonstrates significant effects on modulating the gut microbiota in T2DM-IR rats, but also notably enhances the richness and diversity of this microbiota when administered at a high dose, thereby promoting homeostasis of the core gut microbiota (80). Lycium barbarum polysaccharide, similar to Salvia miltiorrhiza, not only alleviates hyperglycemia, hyperlipidemia, and IR in diabetic mice but also highlights the crucial role of gut microbiota in regulating IR, as evidenced by the negative correlation between the presence of *Cetobacterium*, *Millionella*, *Clostridium*, *Streptococcus*, and *Ruminococcaceae* and HOMA-IR (81). The application of licorice extract demonstrated positive effects, effectively ameliorating various symptoms in diabetic mice in a dose-dependent manner and exerting its effects through gut microbiota remodeling (82). The investigation of Puerariae Lobatae Radix in db/db mice demonstrates its potential to effectively ameliorate the inflammatory damage inflicted upon islet cells and mitigate IR, potentially attributed to its ability to enhance the abundance of specific gut microbiota and regulate the expression of metabolite-associated proteins within the gut microbiota (83). Simultaneously, emerging therapeutic approaches such as targeted probiotic supplementation and fecal transplantation demonstrate potential in modulating the gut microbiota composition to prevent and treat IR in individuals with T2DM (84, 85). In the management of T2DM-IR patients, interventions such as administration of multiple prebiotics, probiotic strains or fecal microbiota transplantation exhibit a substantial amelioration in HOMA-IR indices and exert a favorable impact on glycemic control (86–88). The findings not only validate the application of TCM in treating T2DM, but also underscore its significant potential in ameliorating IR (89, 90).

4 T2DM-IR and gut microbiota-mediated BAs metabolism

Current studies have shown that gut microbiota is involved in the development of T2DM and IR through different mechanisms including imidazole propionate, trimethylamine oxide, short-chain fatty acids, and BAs (91, 92). The gut microbiota, along with its metabolites and nutrients, is continuously transported to the liver through the portal blood circulation. Given that the liver is central to glucose and lipid metabolism, T2DM inevitably results in significant alterations in hepatic metabolites and metabolic pathways (93). Of these mechanisms, the BAs pathway is closely linked to liver function and plays a crucial role in regulating glucose and lipid metabolism balance (44). The gut microbiota plays a pivotal role in the biotransformation and reabsorption of BAs, exerting profound effects on host glucose, lipid, and energy metabolism through co-metabolism with BAs (2, 7). This intricate interplay necessitates a deeper understanding of how the gut microbiota intersects with BA metabolism in the context of T2DM and IR.

4.1 The role of gut microbiota in BAs

The biotransformation function of gut microbiota plays a crucial role in determining the concentration of each component within the total pool of BAs. Variations in gut microbiota community structure can lead to significant alterations in the composition of the BAs pool. The gut microbiota extensively diversify and enhance the biological functions of these compounds through various transformations. Specifically, microorganisms can modify human BAs in four distinct manners, including glycine or taurine uncoupling, dehydroxylation, dehydrogenation, and emisomerization of the cholesterol core

(51). The gut microbiota first fine-tunes BAs metabolism by regulating bile saline lyase (BSH) activity (94, 95). BSH enzyme, as a key enzyme of gut microbiota to modify BAs, is widely distributed in different species of gut microbiota, especially in the genus *Bacteroides (*96). Animal experiments have elucidated the various roles of *Bacteroides* species in BAs metabolism with contrasting effects on metabolic health. On one hand, it has been observed that patients with a higher proportion of *bacteroidetes* in their gut microbiota experience greater improvements in lipid levels, body weight, and IR following acarbose treatment (97). *Bacteroides* species have been shown to ameliorate obesity, IR, and lipid metabolism disorders in both high fat diet-induced obese mice and ob/ob mice through the modulation of LCA and UDCA levels (98). This highlights their potential as beneficial modulators of metabolic dysfunction. On the other hand, a distinct enrichment of the *Bacteroidia* class has been linked to the severity of T2DM and disruptions in glucose homeostasis markers (99). Specifically, high-fat diet-fed mice colonized with *Bacteroides fragilis* show exacerbated glucose intolerance and abolished metformin efficacy in improving glucose tolerance, accompanied by a decrease in intestinal *Bacteroides fragilis* and an increase in glycoursodeoxycholic acid levels (73). Moreover, in a hypothermia animal model study, the administration of aqueous extracts of Aconite was observed to modulate the gut microbiota and BAs metabolism, resulting in an elevation of body temperature in mice. The findings demonstrated that Aconite extracts facilitated the browning process of white adipose tissue and enhanced BAT expression, thereby augmenting glucose energy metabolism. Importantly, these effects were attenuated by depletion of gut microbiota and reduction in BAs levels induced by antibiotic treatment, indicating that Aconite extracts ameliorated T2DM through precise modulation of gut microbiota (100). The gut microbiota's biotransformation of BAs is vital in metabolic health, with variations in its community structure significantly altering BAs composition and impacting metabolic functions.

4.2 The impact of BAs on the intestinal tract

In the intricate gut ecosystem, BAs play an indispensable role as crucial defenders of the chemical barrier. Conjugated BAs, such as taurocholate, possess unique high solubility properties that not only maintain optimal acidic pH balance in the intestinal lumen but also significantly enhance the activity of intestinal digestive enzymes to ensure efficient nutrient absorption (101–103). This physiological process not only establishes a robust chemical defense barrier for intestinal health, but also lays the groundwork for normal intestinal function. However, this delicate balance is significantly disrupted when the body encounters a state of hyperglycemia. Hyperglycemia facilitates bacterial migration and interferes with the normal secretion and metabolism of BAs, resulting in fluctuations in BAs content within the intestinal lumen. BAs directly target lipopolysaccharide and facilitate the decomposition of endotoxin into non-toxic subunits or the formation of micropolymers (104, 105). They modulate the expression of tight junction proteins, thereby bolstering the integrity of the intestinal mucosal barrier and effectively thwarting the invasion of harmful substances (106). BAs contribute to maintaining the balance of the gut microbiota by inhibiting the excessive proliferation of harmful bacteria. This mechanism involves precise regulation of bacterial proliferation, morphology, membrane permeability, and energy metabolism (107, 108). It is important to emphasize that various types of BAs manifest unique biological effects in maintaining intestinal homeostasis (109–111). For instance, CDCA demonstrates remarkable efficacy in repairing inflammation-induced damage to the intestinal epithelium, thereby reversing the observed increase in transepithelial resistance and decrease in expression of tight junction proteins induced by lipopolysaccharide (112). DCA, LCA, and their derivatives effectively prevent heightened intestinal permeability (113). Moreover, UDCA facilitates migration and repair of damaged intestinal epithelial cells while safeguarding the intestine against further harm caused by detrimental substances like lipopolysaccharides (114). In addition, the TCM prescription Tong-Xie-Yao-Fang in the treatment of irritable bowel syndrome has also demonstrated the unique curative effect. The mechanism of Tong-Xie-Yao-Fang involves the regulation of BAs metabolism in the gut, specifically targeting the synthesis and excretion levels of BAs in irritable bowel syndrome rats. This modulation reduces activation of the colonic BAs membrane receptor Takeda G protein-coupled receptor 5 (TGR5) sensing and its mediated Calcitonin gene-related peptide-positive neuronal response were attenuated, thereby contributing to alleviating symptoms associated with irritable bowel syndrome (115). Pien Tze Huang, a Class 1 nationally protected TCM, has the capacity to activate TGR5 via BAs, thereby exerting its anti-inflammatory effects, enhancing intestinal barrier function, and inhibiting pro-inflammatory pathways (116, 117). TCM demonstrates the therapeutic potential of modulating BAs metabolism in maintaining gut health.

5 The gut microbiota-BAs axis affects glucose and lipid metabolism and insulin sensitivity

Diet, genetics, and the environment intricately influence gut microbiota and BAs metabolism, significantly impacting metabolic health. Dietary interventions, particularly focusing on fiber supplementation and specific dietary patterns, show promise in modulating these processes. Dietary factors, especially the Western diet, disrupt the BA profile and contribute to metabolic disorders like T2DM. Conversely, specific dietary interventions can positively influence gut microbiota, BAs metabolism, and metabolic health. The Western diet reduces cecal secondary BAs in mice, but oligofructose supplementation prevents this reduction and elevates 6α-hydroxylated BAs by maintaining key bacteria (118). Oligofructose, a well-recognized soluble fiber, functions as a prebiotic, metabolized by gut bacteria to produce short-chain fatty acids, exerting beneficial effects on glucose lowering, HbA1c, and HOMA-IR in T2DM patients (119). The low-carbohydrate ketogenic diet is commonly used for weight loss. In both an observational study with healthy participants (n = 416) and an

intervention study with overweight or obese individuals following this diet, it was found that the diet reduced the abundance of bacteria encoding BSH in the gut microbiota, such as *Lactobacillus murinus*. This led to increased levels of taurodeoxycholic acid and tauroursodeoxycholic acid in circulation, resulting in reductions in body weight and fasting blood glucose levels (120). In addition, a study reveals that a high-fat diet elevates specific gut bacteria in mice, thereby altering the bile acid pool and triggering inflammation. Non-classic amino acid conjugation of the bile acid cholic acid affects intestinal stem cell replenishment, a process mediated by *Ileibacterium valens* and *Ruminococcus gnavus*, which synthesize this specific conjugate (121). These highlights the potential of dietary interventions in modulating BAs metabolism and improving metabolic health. High intake of fatty animal foods increases BAs secretion and is associated with elevated levels of fecal secondary BAs (122, 123). This change may also enhance glucose-6-phosphate dehydrogenase activity which suggests promotion of the pentose phosphate pathway resulting in reduced equivalents (124). The examples provided demonstrate the intricate relationship between diet, gut microbiota, and metabolic processes, highlighting the potential of targeted dietary strategies in mitigating metabolic disorders.

Understanding the genetic and environmental influences on the gut microbiota and BAs metabolism is crucial for developing effective strategies to maintain or improve metabolic health. Building on the observed correlation between gut microbiota structural variation of genome and BAs levels, recent research by Meng et al. further elucidates how antibiotic-induced gut dysbiosis impacts host transcriptome and m(6)A epitranscriptome through BAs metabolism (125, 126). In addition to intrinsic genetic factors, extrinsic environmental factors also exert profound effects on the gut-liver axis, particularly through their interaction with the gut microbiome. Environmental toxins have been linked to gut microbiome dysbiosis and IR, with microbiome-derived secondary BAs potentially acting as mediators between environmental toxins and obesity or IR (127). Lifestyle-induced weight loss does improve glycemic control and IR but does not affect BAs concentrations (128). However, during weight regain, the gut microbiota and BAs play pivotal roles, and supplementation with Parabacteroides distasonis and non-12α-hydroxylated BAs can effectively mitigate weight rebound (129). Thus, understanding these interactions is essential for developing strategies to improve metabolic well-being.

Metabolomics is a potent tool for the precise analysis of metabolites at the molecular level, uncovering disease-induced alterations in metabolites. Monitoring the composition of the human gut microbiota or gut metabolites may provide valuable insights into the diagnosis and treatment of T2DM (130). The BAs pool consists of a variety of BAs subtypes, each with its own unique affinity for different BAs receptors. As a result, BAs play a crucial role in regulating host health by interacting with receptors of varying affinities (131). This interaction not only facilitates the reduction of bile salt load, but also significantly enhances insulin sensitivity, thereby regulating glucose metabolism and controlling energy expenditure (132). The serum BAs concentration is significantly higher in patients with T2DM compared to non-diabetic individuals, and it shows a positive correlation with IR (133). Alessandro et al. analyzed plasma samples from 224 patients with T2DM and 102 nondiabetic individuals with metabolic syndrome, assessing a total of 14 plasma BAs species. This study demonstrates significant disparities in plasma BA profiles between individuals with and without T2DM (134). Chen et al. examined serum samples from 30 individuals and fecal matter from 15 patients to investigate changes in BAs and gut microbiota within the biological milieu of T2DM patients. Their findings reveal a dynamic interaction between BAs and gut microbial populations, highlighting notable elevations in DCA, LCA, and glycodeoxycholic acid levels among the T2DM group, while glycoursodeoxycholic acid levels were significantly reduced (135). Significant changes were reported in the levels of BAs metabolites in the liver of T2DM-IR mice (136). T2DM with IR often presents with inflammation and liver damage (137). Low-level inflammation is thought to be a key factor in the pathogenesis of IR, T2DM, and beta cell impairment (138). Research has demonstrated significant differences in the serum BAs profiles between healthy individuals and patients with intestinal inflammation (109). Taurocholate Deoxycholate has demonstrated efficacy in mitigating inflammation and enhancing insulin sensitivity (139, 140). Subsequent to continuous administration of glycoursodeoxycholic acid, a notable decrease in blood insulin levels and HOMA-IR was observed in db/db mice (34). It is important to note that moderate BAs supplementation can significantly reduce inflammation in the body and effectively improve insulin sensitivity (141, 142).

Bear bile has been used in TCM for thousands of years (143). UDCA, the active ingredient in bear bile, was found to have metabolic, anti-inflammatory, and antioxidant effects in patients with T2DM in a prospective, double-blind, placebo-controlled clinical study (144, 145). Scutellaria baicalensis, a TCM, has been found to modulate the profile of BAs in the intestine through its total flavonoid components, which not only shapes the composition of the intestinal microbiome by promoting the growth of beneficial bacteria and inhibiting harmful bacterial proliferation, thereby optimizing BAs metabolism, but also enhances intestinal barrier function and reduces inflammation caused by BAs accumulation, potentially alleviating IR in T2DM patients (146). Furthermore, Forsythia, a traditional Chinese medicinal herb renowned for its therapeutic properties including clearing heat, detoxification, reducing inflammation, and dispelling wind-heat, has been subject to recent research revealing that its active compound, Phillyrin, shows promise in improving IR (147). Additionally, the study indicates that, when compared to green Forsythia, mature Forsythia may exert more pronounced effects on detoxification and BAs metabolism. This intriguing discovery implies that Forsythia, through its influence on the gut microbiota, could indirectly regulate BAs metabolism, which would ultimately lead to a positive impact on IR in patients with T2DM (148). In a retrospective cohort study involving 147 patients, the Jiang-Tang-San-Huang pill demonstrated significant efficacy in lowering blood glucose levels and reducing IR, thereby enhancing pancreatic islet cell function (149). The therapeutic effects of Jiang-Tang-San-Huang pill are mediated through the correction of gut microbiota imbalances, particularly by enriching bacteria with BSH activity,

including *Bacteroides*, *Lactobacillus*, and *Bifidobacterium*. This enrichment facilitates the accumulation of unconjugated BAs in the ileum (150). Ultimately, the Jiang-Tang-San-Huang pill alleviates T2DM-IR by modulating the complex interplay between gut microbiota and BAs metabolism. The research conducted on Jingangteng capsule in diabetic rat models demonstrated that it possesses a significant ability to regulate BAs metabolites linked to BAs receptors and reverse the diminished expression of BAs receptors in the liver. This regulatory mechanism not only downregulates the expression of lipogenesis-related genes but also inhibits the activation of inflammation-related genes, actions which collectively contribute to reducing the inflammatory burden on the liver and intestines, thereby improving IR in patients with T2DM (151). Ji-Ni-De-Xie, a traditional herbal formulation utilized in Tibetan medicine for treating T2DM, has exhibited notable effects in ameliorating IR through modulation of BAs metabolism. The study indicates that Ji-Ni-De-Xie can adjust the composition and concentration of BAs in the intestines, optimize their distribution and function, reduce potential damage to the intestinal mucosa, and enhance nutrient absorption and utilization (152). It's worth noting that electroacupuncture can improve the disorder of glucose and lipid metabolism in db/db mice, increase the abundance of the *Firmicutes* and *Actinobacteria*, and elevate the content of fecal BAs pool, particularly CA and UDCA (132). These comprehensive mechanism effectively alleviates IR, hyperglycemia, hyperlipidemia, and inflammatory responses, offering new scientific evidence for integrating traditional Chinese medicine into T2DM treatment. Relevance of gut microbiota-mediated BAs metabolism in the development of IR in individuals with T2DM in Figure 2.

6 T2DM-IR and BAs-related signaling pathways

BAs are a unique class of signaling molecules that can activate a variety of nuclear and membrane receptors (153). Over the past three decades, the scientific community has conducted extensive and comprehensive research into the regulatory functions of BAs receptors in maintaining health and driving disease progression (9, 55, 154). In recent years, studies have revealed that BAs exhibit remarkable efficacy in lowering blood glucose levels through multiple signaling pathways (140, 155, 156). Specifically, it is through the activation of key receptors, such as FXR and TGR5, that BAs profoundly influence numerous physiological processes, which in turn encompass the stimulation of GLP-1 secretion in the intestine, the inhibition of hepatic gluconeogenesis, an increase in energy expenditure, and the modulation of inflammatory responses

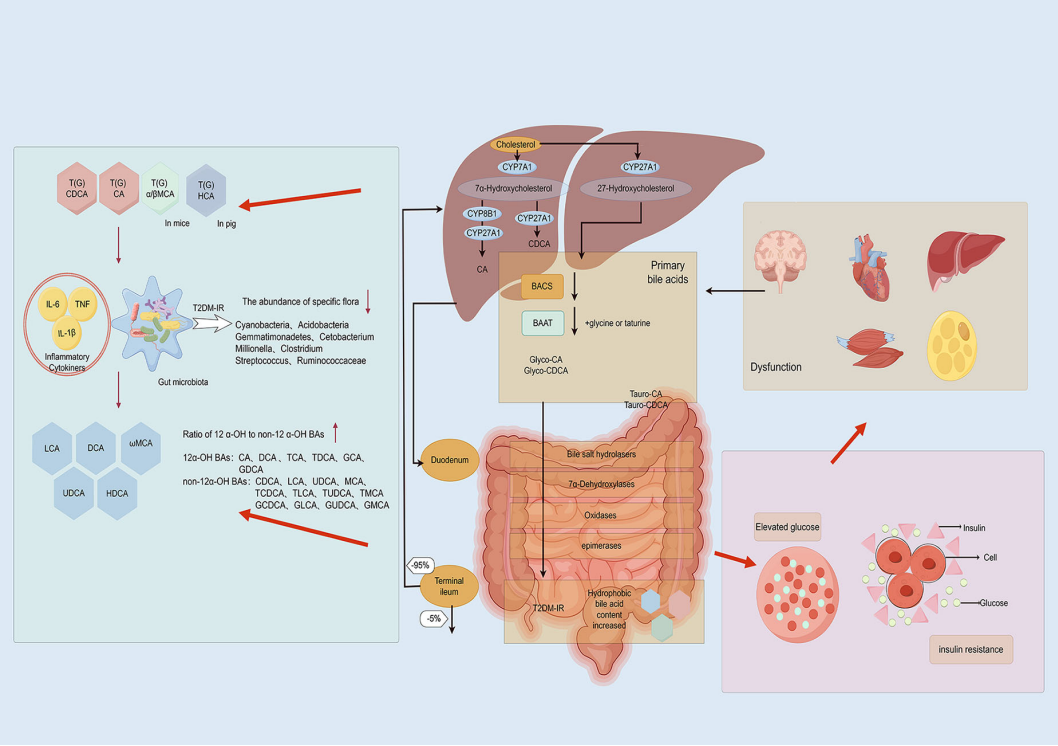

FIGURE 2
Relevance of gut microbiota-mediated BAs metabolism in the development of IR in individuals with type 2 diabetes mellitus(By Figdraw). Patients with type 2 diabetes mellitus often exhibit intestinal inflammation, resulting in increased intestinal permeability that allows bacterial lipopolysaccharides and BAs to directly enter the liver through the portal vein. Within the intestinal microecosystem, a series of biochemical processes such as uncoupling, dehydrogenation, dehydroxylation, and differential isomerization convert BAs into secondary BAs. As the primary organ responsible for BAs synthesis and metabolism, the liver may alter its synthesis and secretion under inflammatory stimulation, thereby influencing the metabolic balance of BAs. Disordered BAs metabolism can lead to elevated glucose levels and IR, further exacerbating pathological progression.

and gut microbiota composition (157, 158). All of these effects play significant roles in the pathogenesis of IR and T2DM (159, 160).

6.1 Membrane receptor TGR5

TGR5, a crucial membrane receptor for BAs, exhibits high expression in the gallbladder, liver, intestinal tract, and adipose tissues. Functioning as a BAs receptor, TGR5 can respond to all known BAs irrespective of their binding state. Studies have shown that secondary BAs exhibit markedly higher affinity for TGR5 than primary BAs, with LCA emerging as the most potent natural agonist for TGR5 (159, 161). Activation of this receptor demonstrates anti-inflammatory properties and aids in preventing chronic inflammation (162). Inflammatory factors have the potential to disrupt insulin signaling pathways through endocrine or paracrine mechanisms, thereby impacting its transduction activity and ultimately leading to T2DM-IR (163).

In the liver, TGR5 is predominantly localized in sinusoidal endothelial cells, Kupffer cells, and cholangiocytes (164). Upon bacterial infiltration into the liver via bloodstream, their surface antigens can trigger macrophage activation leading to the production of proinflammatory cytokines including interferon-γ, interleukin-1β, interleukin-6, and tumor necrosis factor-α. These cytokines promote polarization of M0 macrophages towards a proinflammatory M1 phenotype (165). However, activation of TGR5 has been shown to inhibit nuclear factor kappa-B (NF-κB) signaling pathway thereby attenuating inflammation in Kupffer cells and facilitating transition from an M1 proinflammatory phenotype to an anti-inflammatory M2 phenotype (166, 167). Notably, NF-κB signaling pathway represents a pivotal regulatory axis governing immune response gene expression closely associated with IR in T2DM (168). Qi et al. revealed that taurochenodeoxycholic acid (TCDCA) exerts critical functions in apoptosis and immune responses via the TGR5 receptor, ultimately regulating NF-κB activity (169). In the intestine, cyclic adenosine monophosphate (cAMP) acts as a secondary messenger and is positively regulated by TGR5, leading to rapid and dose-dependent elevation of cAMP levels. Activation of TGR5 leads to an increase in cAMP levels, subsequently stimulating protein kinase A (PKA) and inducing NOD-like receptor family pyrin domain-containing 3 (NLRP3) ubiquitination, which ultimately inhibits NLRP3 inflammasome activation. The NLRP3 inflammasome is pivotal in chronic inflammation and is intimately linked to insulin signaling, glucose tolerance, and IR (170). This mechanism was validated in a mouse model, where BAs were found to alleviate various inflammatory diseases, including high-fat diet-induced T2DM (154). Notably, a modified Gegen Qinlian decoction has been reported to effectively regulate gut microbiota, improve BAs metabolism, and activate the TGR5/cAMP/PKA signaling pathway, thereby exerting therapeutic benefits in mouse models of T2DM (171). Moreover, the research expounded upon the robust connection between TGR5 and GLP-1. Intriguingly, approximately 75% of GLP-1-generating intestinal endocrine cells within isolated human intestinal cells demonstrated expression of TGR5 (156). The activation of TGR5 instigated the discharge of GLP-1 from intestinal endocrine cells, subsequently activating the GLP-1R/PKA signaling pathway and markedly reducing the HOMA-IR index (172, 173). As a crucial intermediary molecule, the influence of GLP-1 transcended the intestine and encompassed various body regions, effectively mitigating peripheral IR (174). In adipocytes, elevated levels of TCA and DCA could activate TGR5. Upon activation, TGR5 stimulated UCP1 expression, thereby fostering increased heat production in white fat and contributing to the improvement of IR (41, 175, 176). The research further substantiated an inverse correlation between UCP1mRNA content and IR, specifically indicating that a higher content corresponded to a lower degree of IR (177). AMPK, a kinase activated by PKA, has been implicated in the onset of metabolic syndrome, including obesity and hyperglycemia (178). Vaccarin, a flavonoid derived from vaccaria seeds and traditionally employed in Chinese medicine to promote blood circulation, has recently been demonstrated to exert anti-diabetic effects (179). It has been demonstrated that vaccarin stimulates the growth of *Bacteroides uniformis* both *in vitro* and *in vivo*, resulting in the production of CA and CDCA that act through the TGR5/AMPK signaling pathway to inhibit hepatic gluconeogenesis and lipolysis, thereby ameliorating diabetes and its potential complications (180). The findings of this study not only enhance the understanding of the intricate relationship between TGR5 and IR but also provide new insights into potential therapeutic interventions for treating IR-related disorders, such as T2DM.

6.2 Nuclear receptor FXR

As a crucial nuclear receptor, FXR is predominantly expressed in the intestinal and hepatic tissues, playing an indispensable role in maintaining homeostasis of BAs, lipids, glucose, and energy (181). Natural agonists of FXR such as CA and CDCA have been demonstrated to activate its function (182). In the db/db mouse model of T2DM characterized by IR, treatment with BAs or specific agonists for FXR significantly improved insulin sensitivity and effectively reduced blood glucose levels thereby ameliorating diabetic symptoms (183). The FXR receptor plays a crucial role in the regulation of diverse metabolic pathways, encompassing BAS synthesis, excretion, reverse cholesterol transport, gluconeogenesis, and glycogenolysis (95, 184, 185).

Increased expression or transcriptional activity of FXR in the liver led to a significant reduction in blood glucose levels and improvement in IR, whereas surprisingly, absence of FXR expression or inhibition of its transcriptional activity in the intestine also resulted in decreased blood glucose levels and improved IR (186, 187). This discovery unveils the intricate regulatory mechanism of FXR across different tissues. The FXR/SHP and FXR/FGF15/19/FGFR4 pathways have been identified as the principal negative regulatory pathways in BAs synthesis (133). In intestinal tissues, FXR stimulates the synthesis of fibroblast growth factor 15/19(FGF15/19)and effectively inhibits the expression of CYP7A1 and CYP8B1, thereby regulating BAs synthesis. As an antagonist of FXR, UDCA significantly inhibits the transcription activity of FXR induced by CDCA, highlighting

the crucial role of FXR in metabolic regulation (73). By suppressing the signaling pathway of FXR, the release of FGF15/19 is diminished, thereby impeding its binding to fibroblast growth factor receptor 4 (FGFR4) on hepatocytes via the portal vein. This attenuation weakens the inhibitory effect on CYP7A1 and CYP8B1, resulting in an elevation in hepatic BAs synthesis and cholesterol utilization. Consequently, this phenomenon contributes to ameliorating obesity and IR (158). Berberidis Cortex, a traditional Tibetan herbal remedy, ameliorates BAs dysregulation in T2DM rats, notably by elevating levels of TCDCA and CA, subsequently activating the FXR/FGF15 signaling pathway and inhibiting hepatic gluconeogenesis (188).

Another significant aspect is increased hepatic BAs levels and FXR activation induce the expression of small heterodimer partner (SHP) gene which inhibits transcriptional activity of CYP7A1, a key enzyme involved in BAs production, thereby enhancing fatty acid oxidation via peroxisome proliferator-activated receptor alpha (PPARα) activation (189–191). Activation of PPARα leads to reduced blood lipid levels and plays a crucial role in glucose homeostasis as well as IR management (192). In a rat model of T2DM, Roux-en-Y gastric bypass surgery elevated serum BAs levels, which subsequently activated the FXR-related pathway, leading to upregulation of FXR, SHP, and PPARα expression that inhibited adipogenesis and modulated hepatic gluconeogenesis (193). Recent investigations further elucidate the intricate interplay of these pathways in modulating glucose metabolism and insulin sensitivity, exemplified by a study demonstrating that a degraded sweet corn cob polysaccharide ameliorates T2DM through the modulation of BAs synthesis and hepatic lipid metabolism, a mechanism that involves the activation of the hepatic FXR/SHP pathway and the engagement of the FXR/FGF15/FGFR4 signaling axis within the gut-liver axis (194). Furthermore, SHP, a downstream target gene regulated by FXR, has been shown to inhibit NLRP3 inflammasome formation along with downstream inflammatory factors resulting in decreased IR (170, 195). A novel fasting therapy has been found to ameliorate high-fat diet- and STZ-induced IR in rats by suppressing NLRP3 inflammasome-mediated inflammatory response (196). In addition, the role of GLP-1 warrants attention. Oral administration of UDCA not only stimulates GLP-1 secretion but also effectively improves symptoms related to glucose intolerance and IR by inhibiting the FXR signaling pathway (197). Similarly, a single-center cohort study revealed that intestinal Clostridium inhibits the conversion of CDCA to UDCA, leading to a reduction in GLP-1 secretion and subsequently resulting in abnormal glucose metabolism and IR (197). In intestinal L-cells, FXR disrupts the interaction between glucose-responsive transcription factor carbohydrate-responsive element-binding protein (ChREBP) and its target sequences, thereby inhibiting glycolysis and resulting in a reduction in proglucagon expression and, consequently, a decrease in GLP-1 secretion (198).

Nevertheless, studies have demonstrated an intricate interplay between the bile acid receptors FXR and TGR5. Curcumin and Notoginsenoside Ft1, acting as a natural TGR5 agonist and an intestinal FXR inhibitor, exerts a dual effect that alleviates metabolic disorders by activating TGR5 while simultaneously suppressing FXR (199, 200). Additionally, the traditional medicinal formula Jiang-Tang-San-Huang pill upregulates the intestinal FXR/FGF15 and TGR5/GLP-1 signaling pathways (150). In another context, Mulberry leaf polysaccharides have been shown to significantly reduce fasting blood glucose and lipid levels, improve glucose and lipid metabolism, and mitigate IR, and these effects are mediated through the modulation of BAs metabolism, as evidenced by increased ileal expression of TGR5 and suppressed hepatic and ileal expression of FXR mRNA in T2DM rats (201). Collectively, these findings underscore the therapeutic potential of targeting the FXR/TGR5 axis through natural compounds and traditional medicines for the management of metabolic diseases. FXR and TGR5 are summarized in Figure 3.

6.3 Other receptors

The liver, as the central organ for glucose metabolism, plays a crucial role in maintaining energy homeostasis within the body. In recent years, pregnane X receptor (PXR), a member of the nuclear receptor superfamily, has been identified to be involved in regulating hepatic sugar metabolism and is closely associated with the development of obesity and IR (202, 203). PXR exhibits abundant expression in both the liver and intestine, where it governs the expression of downstream target genes (204). The activation of PXR receptors was observed to induce an up-regulation of the lipid receptor Peroxisome proliferators-activated receptors γ in the liver, thereby promoting hepatocyte steatosis (205). This effect further exacerbates hepatic burden and may lead to hepatic IR. Furthermore, PXR knockout mice fed a high-fat diet exhibit inhibited weight gain, hepatic steatosis, IR, and gluconeogenesis (206). The Vitamin D Receptor (VDR) receptor, which is highly expressed in islet β cells, plays a significant role in reducing islet inflammation, protecting β cell function, and preventing T2DM (207). Moreover, activation of the VDR receptor in liver macrophages not only alleviates inflammation and liver steatosis but also improves insulin sensitivity and effectively addresses IR (208).

7 Limitation

BAs, due to their intricate associations with lipid and glucose metabolism, have emerged as a focal point in the realm of metabolic disease research. Extensive studies have eloquently elucidated the pivotal role of BAs in glycolipid metabolism, and a thorough dissection of their physiological and pathological alterations is paramount to unraveling the underlying mechanisms of IR and T2DM (130, 158, 209). Nonetheless, the exploration of the impact of BAs on glycemic responses in both healthy and diseased states in humans remains fraught with numerous challenges and limitations especially at the level of TCM research. BAs are not a singular molecular entity but rather a complex system comprised of diverse metabolites, each possessing distinct biological activities. The mechanisms of action of these metabolites in glucose metabolism are varied and, in part, remain uncharted. Secondly, species differences pose another significant barrier. Fundamental disparities in BAs biology exist between mice and humans,

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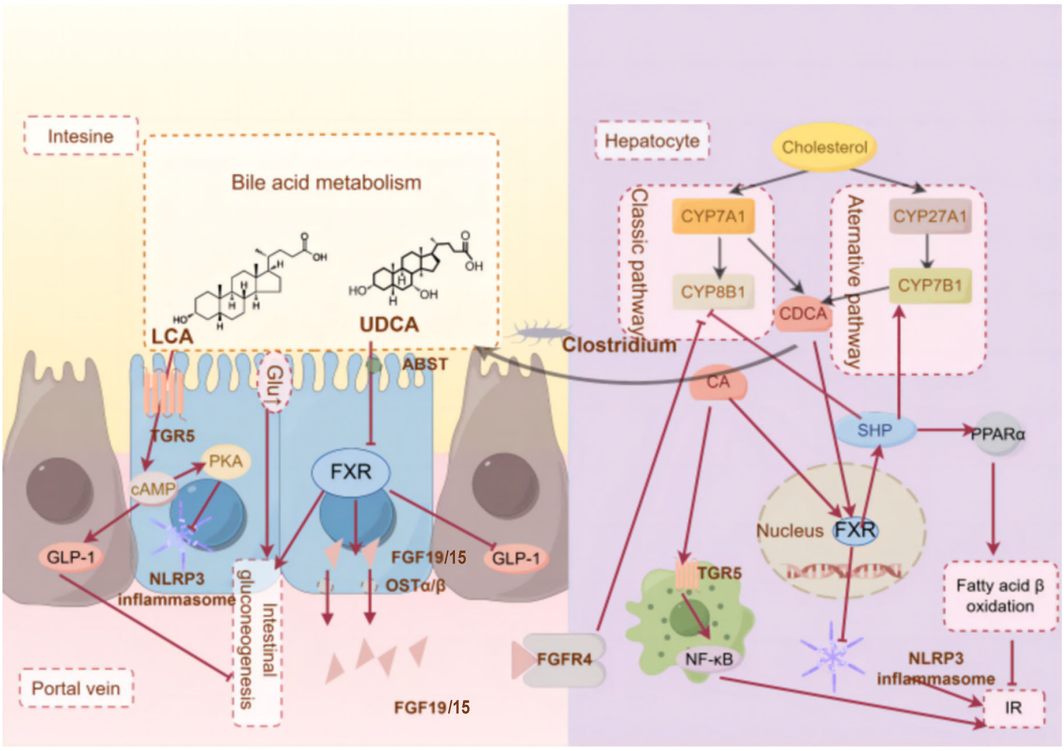

FIGURE 3
The molecular mechanism of BAs receptors ameliorate T2DM-IR.(By Figdraw)FXR and TGR5 are key BAs receptors that are highly expressed in tissues such as the liver and intestine, responding to bile acid pool composition and inhibiting inflammation, helping to prevent insulin resistance. These metabolites can act on the gut and liver through different signaling pathways, thus triggering different physiological and biochemical reactions.

complicating the direct translation of findings from mouse models to human applications (63). This cross-species divergence amplifies the difficulty in converting experimental results into clinical practices. Furthermore, the intricate individual variability of the human gut microbiome profoundly influences the diversity of gut microbial community structures, subsequently shaping unique BAs metabolic profiles (37). This variability serves as a significance for the progression of diseases and the variability in responses to BAs regulatory therapies. This phenomenon closely aligns with the concept of "constitution" in TCM, reflecting deep-seated differences in the physiology and pathology of individuals (210). In the face of challenges posed by individual variability in the practical application of BAs-related treatment strategies, TCM, with its unique approach to constitutional identification and treatment philosophy, offers new perspectives and possibilities for precision medicine. While TCM demonstrates potential in modulating gut microbiota and influencing BAs metabolism, evidence for the effective translation of animal model findings to human clinical trials is currently lacking. This underscores the need for enhanced interdisciplinary collaboration and optimized research designs in future studies to better evaluate the efficacy and safety of TCM in treating T2DM and IR through the gut microbiota-BAs axis. In summary, despite some progress, research on BAs as a key target for treating T2DM faces numerous limitations. Future studies must adopt more precise and personalized strategies to address these challenges, driving further advancements in this field.

8 Conclusions

The regulatory role of BAs in the gut-liver axis is of central importance for alleviating IR in T2DM. Current research has delved into the roles of the bile acid nuclear receptor FXR and the membrane receptor TGR5 in T2DM, with some studies in TCM also focusing on this area, emphasizing the potential of TCM in regulating BAs metabolism. Despite these advancements, the specific alterations in BAs levels, abnormalities in their composition, and their association with the pathogenic mechanisms in T2DM remain to be thoroughly explored. TCM boasts a long history and remarkable efficacy in the treatment of T2DM, highlighting holistic regulation and multi-target synergistic effects. Regrettably, in the realm of TCM research targeting T2DM through BAs regulation, there is a notable dearth of adequate modern scientific validation. Notably, the holistic and dynamic nature emphasized by metabolomics research on BAs and gut microbiota aligns well with the "concept of holism" in TCM, offering the potential for scientific explanations of multi-target and multi-pathway mechanisms of action. By taking the BAs metabolic pathway as an entry point and dissecting the complex mechanisms of TCM in T2DM treatment at a microscopic level, we may gain new research perspectives and methodologies, fostering deeper integration of Chinese and Western medicine. There is an urgent need to identify more effective strategies that precisely regulate and utilize BAs metabolism to improve IR in T2DM

patients, thereby paving new avenues for the prevention and treatment of this global disease and significantly enhancing health status and quality of life.

Author contributions

YW: Conceptualization, Writing – original draft. JY: Writing – original draft. BC: Investigation, Writing – original draft. WJ: Investigation, Writing – original draft. MW: Visualization, Writing – original draft. XC: Supervision, Writing – original draft. MJ: Visualization, Writing – original draft. LS: Writing – review & editing. CP: Funding acquisition, 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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A systematic review on gut microbiota in type 2 diabetes mellitus

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Aims/hypothesis: The gut microbiota play crucial roles in the digestion and degradation of nutrients, synthesis of biological agents, development of the immune system, and maintenance of gastrointestinal integrity. Gut dysbiosis is thought to be associated with type 2 diabetes mellitus (T2DM), one of the world's fastest growing diseases. The aim of this systematic review is to identify differences in the composition and diversity of the gut microbiota in individuals with T2DM.

Methods: A systematic search was conducted to identify studies reporting on the difference in gut microbiota composition between individuals with T2DM and healthy controls. Relevant studies were evaluated, and their characteristics and results were extracted using a standardized data extraction form. The studies were assessed for risk of bias and their findings were reported narratively.

Results: 58 observational studies published between 2010 and 2024 were included. Beta diversity was commonly reported to be different between individuals with T2DM and healthy individuals. Genera Lactobacillus, Escherichia-Shigella, Enterococcus, Subdoligranulum and Fusobacteria were found to be positively associated; while Akkermansia, Bifidobacterium, Bacteroides, Roseburia, Faecalibacteirum and Prevotella were found to be negatively associated with T2DM.

Conclusions: This systematic review demonstrates a strong association between T2DM and gut dysbiosis, as evidenced by differential microbial abundances and altered diversity indices. Among these taxa, *Escherichia-Shigella* is consistently associated with T2DM, whereas *Faecalibacterium prausnitzii* appears to offer a

protective effect against T2DM. However, the heterogeneity and observational nature of these studies preclude the establishment of causative relationships. Future research should incorporate age, diet and medication-matched controls, and include functional analysis of these gut microbes.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42023459937.

KEYWORDS

gut microbiota, diabetes, gut dysbiosis, diabetes mellitus, systematic review

1 Introduction

The human body hosts a vast population of microorganisms, including archaebacteria, viruses, fungi and eubacteria (also referred to as bacteria), collectively referred to as microbiota. The period of initial gut colonization in humans remains a contentious topic, with some studies suggesting such colonization occurs *in utero*, while others refute this suggestion. Regardless, it is widely accepted that in humans, the infant gut microbiota is rapidly populated near the time of birth, typically achieving stability between the ages of 2 and 5 (1).

Due to factors such as peristalsis, pH, oxygen and biological products, the microbiota varies throughout different parts of the gastrointestinal tract. The small intestine contains fewer microorganisms due to a faster transit time, acidic environment, and the presence of bile and pancreatic secretions. In contrast, the large intestine hosts billions of microorganisms, mainly dominated by anaerobic bacteria, including Firmicutes, Bacteroides, Actinobacteria, Proteobacteria and Verrucomicrobia (2). This is the primary site where the microbiota interact with the human host (3).

Gut microbiota are involved in core human bodily functions including digestion and nutrient degradation, synthesis of biological agents, immune system development and maintenance of gut integrity (4). Significant factors that influence the microbiotia gut composition include age, gender, geographical location and diet. Additionally, prebiotics and probiotics have been used to change the composition of gut microbiota and induce beneficial effects. It has also been suggested that early microbial transfer during the formation and development of the gut microbiota may play a role in the inheritability of human conditions such as neurological diseases and obesity (5).

The gut bacterial microbiome has been associated with the pathophysiology of multiple chronic diseases, one of which is Type 2 diabetes mellitus (T2DM) (6–8). Type 2 diabetes mellitus (T2DM) is characterized by chronic hyperglycemia due to decreased insulin secretion by pancreatic beta cells and increased insulin resistance. Rapid urbanization, nutrition transition and sedentary lifestyles have led to a drastic rise in cases (9). In 2018 there were over 500 million cases of T2DM globally (172). In Australia, the number of patients with T2DM increased to 1 million accounting for 2.3% ($2.7 billion AUD) of total disease expenditure in 2015-2016.

Increasing evidence shows that alterations in gut bacterial microbiota plays a crucial role in the development of T2DM. Gut bacterial dysbiosis in individuals with T2DM is thought to cause systemic inflammation and altered metabolism, leading to increased peripheral insulin resistance (4). Over time, this can lead to the development of complications such as diabetes related foot complications. Hence, it is crucial to identify bacteria contributing to the development and exacerbation of this disease, as well as those that play a protective role in preventing it.

2 Aim of systematic review

Several studies have established that the composition and function of gut bacterial microbiota in individuals with T2DM are different from healthy individuals. Despite this, the specific microbial changes remain largely unknown. This systematic review aims to provide an updated review on whether the gut bacterial microbiota profile of individuals with T2DM differ from healthy individuals. Mechanisms contributing to the pathophysiology of T2DM will also be discussed.

3 Methods

3.1 Search strategy

We performed a detailed systematic review of published data according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta Analyses) guidelines. The methodological approach was registered in PROSPERO (International prospective register of systematic reviews) database under protocol number CRD42023459937.

Embase and PubMed literature search was performed on articles between Jan 1st 2010 and April 15th 2024. The search strategy combined MESH (Medline) and free terms using the

boolean operators "AND" and "OR". "Diabetes Mellitus", "gut microbiome", "intestinal flora" and "gastrointestinal microbiome" were terms used in the search. A complementary search was carried out in the references of studies included. The search protocol is shown below:

((("Diabetes Mellitus"[Majr: NoExp] OR "Diabetes Mellitus, Type 2"[Majr: NoExp] OR T2D[Text Word] OR type 2 diabetes [Text Word] OR "type 2 diabetes mellitus"[Title/Abstract:~2]) AND ("Gastrointestinal Microbiome"[Majr] OR gut micro*[Text Word] OR intestine flora[Text Word] OR intestinal flora[Text Word] OR gut flora[Text Word] OR intestine micro*[Text Word] OR intestinal micro*[Text Word] OR Gastrointestinal micro*[Text Word])) NOT (animals[Mesh] NOT humans[Mesh])

3.2 Eligibility criteria

All original peer reviewed research publications were considered. Eligible studies included observational human studies specifically examining gut microbiota in T2DM patients compared with control groups.

Exclusions: studies on type 1 diabetes mellitus or gestational diabetes; those without control groups; longitudinal studies; studies on children or adolescents aged <18 years or in the elderly aged >80 years; non-English studies; studies with only abstracts available; and studies with high risk of bias.

Microbial taxa were defined as positively or negatively associated with T2DM if p value <0.05 when comparing taxa abundance between individuals with T2DM and healthy controls. For linear discriminant analysis (LDA), a score of >4 indicated a positively association, while <4 indicated a negative association. For prospective studies with interventions, the baseline result was used. For studies with more than one population group, results were only reported to be positively or negatively associated if both groups demonstrated the result. Microbial taxa without reported p values, p values >0.05 or LDA values <4 were classified as non-significant and into an increased, decreased or equivocal (equal abundance or not reported) trend.

The titles and abstracts of all identified studies were reviewed by two independent authors. Studies were assessed using the Newcastle–Ottawa Quality Assessment Scale. This instrument included three domains: selection, comparability, and outcomes. High risk of bias was determined when some of the domains did not receive a point, in which case that study was excluded. Ambiguities in selection criteria were resolved by discussions between at least 3 researchers.

3.3 Data extraction

The data extracted from the studies included in this systematic review are summarized in Supplementary Table 1 with the following information: author and year of publication, country and period of study/seasons (if available), sample size and characterization of the study population, method used to evaluate the gut microbiota and bacteria analyzed (if applicable), and outcomes.

4 Results and discussion

In total, 58 human observational studies were included in this review (Figure 1). The majority of these studies reported associations between specific taxa and the development and exacerbation of T2DM. However, no taxa were universally agreed upon to be positively or negatively associated with T2DM.

4.1 Alpha and beta diversity

4.1.1 Alpha diversity

Alpha diversity refers to the microbial species diversity (richness) within a functional community. Reported indices included the Shannon index, Chao1 index, Simpson index, Faith index, Observed index, Abundance-based Coverage Estimator (ACE) index and Good's Coverage. The Shannon index was the most commonly reported metric. A p value of <0.05 was deemed statistically significant. Most analyses reported no difference in alpha diversity between T2DM individuals and healthy controls (Table 1). Alpha diversity metrics varied by ethnicity, oral antihyperglycemic agents and other environmental factors (20, 31,

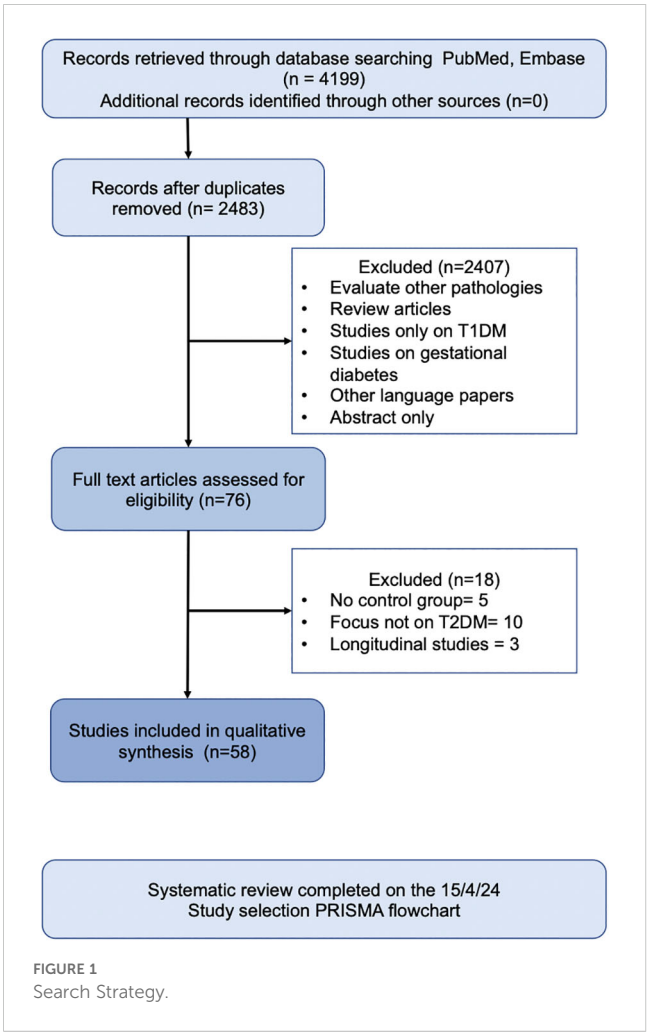

FIGURE 1
Search Strategy.

TABLE 1 PMID of studies reporting alpha diversity indices in type 2 diabetes compared to controls.

Alpha diversity Indices	Increased in T2DM	Reduced in T2DM	No significant difference
Shannon	(10, 11)	(12–18)	(19–33)
Chao 1	(10)	(14, 18, 27, 34, 35)	(13, 21, 25, 26, 30, 31, 36, 37)
Simpson		(12)	(25–28, 31)
Faith	(10)	(13, 34)	(21, 22, 36)
Observed	(10, 11)	(16, 21)	(20, 22, 25, 26, 29, 31, 36, 38)
ACE		(27)	(25, 26, 29, 31)
Good's Coverage		(36)	(25)
Unspecified			(39)

40). Higher diversity was observed in treatment naïve T2DM individuals compared to those receiving treatment (41).

4.1.2 Beta diversity

Beta diversity describes the amount of differentiation and dissimilarities between gut bacterial microbiota communities. The most common beta diversity metric used was the unweighted Unifrac distance. A p value of < 0.05 was deemed significant. The majority of studies reported a significant difference in beta diversity in individuals with T2DM compared to healthy controls (Table 2).

4.2 Phylum analysis - prevalence of firmicutes, bacteroidetes and the firmicutes/bacteroidetes ratios

This review focuses on the phylum and genus levels of gut bacteria. The human gut bacterial microbiota consists mainly of Firmicutes and Bacteroidetes, which make up over 90% of the community. The remaining 10% includes phyla like Proteobacteria, Actinobacteria and Verrucomicrobia. In individuals with T2DM, the most commonly altered phyla are Firmicutes and Bacteroidetes (Figure 2, Table 3).

4.2.1 Firmicutes and bacteroidetes

Overall, an unchanged Firmicutes and reduced Bacteroidetes abundance were observed among individuals with T2DM.

TABLE 2 Summary of studies reporting beta diversity in type 2 diabetes compared to controls.

	Significant difference	Difference	No significant difference
Beta Diversity	(10, 13–17, 22, 27, 34–36, 39, 41)	(21, 25, 37)	(11, 19, 23, 24, 26, 29, 30, 38, 42)

If difference in beta diversity was observed but no p values were reported, they were classified as having difference.

TABLE 3 Summary of studies reporting Firmicutes and Bacteroidetes abundance.

	Increase	Decrease	No significant difference
Firmicutes	(18, 21, 34, 36, 41)	(11, 13, 35, 37, 43–46)	↑ (19, 25, 26, 30, 33, 38) ↓ (14, 16, 24, 39) Equivocal (47)
Bacteroidetes	(11, 35, 44)	(12, 16, 18, 34, 36, 38, 39, 41, 48)	↑ (14, 37) ↓ (13, 19, 21, 25, 26, 30, 33, 43) Equivocal (24)

Studies with no significant differences are reported as trends. ↑ - increase, ↓ - decrease.

An unchanged Firmicutes abundance may be due to a simultaneous increase in opportunistic Firmicutes pathogens such as *Enterococcus* (Table 4), *Eisenbergiella* (16) *Acidaminococcus* (29, 41) and a decrease in beneficial Firmicutes microbes including *Faecalibacterium* and *Roseburia* (Table 5)

Meanwhile, Bacteroidetes are thought to be beneficial to human health with several genera including *Bacteroides* and *Prevotella* considered an untapped resource for next-generation prebiotics. Both these taxa, proposed to mitigate metabolic endotoxaemia and inflammation, were reduced among individuals with T2DM (Table 5). Bacteroidetes have negative correlation with fasting blood glucose levels (27, 36), corresponding with their reduced levels in T2DM.

4.2.2 The firmicutes/bacteroidetes ratio

The Firmicutes/Bacteroidetes (F/B) ratio (Table 6) represents the relationship between two dominant phyla and is commonly used as a marker of gut dysbiosis.

The F/B ratio was not consistently associated with clinical parameters. Larsen et al. found a positive correlation between the Bacteroidetes to Firmicutes ratio and plasma glucose (37) while Wang et al. reported a positive correlation between the F/B ratio and body mass index (BMI), fasting blood glucose levels and HBA1c (27). Other studies found no correlation with fasting, postprandial blood glucose levels (30), age, HBA1c or lipid profile (39). This suggests that while the F/B ratio indicates dysbiosis, it does not specifically predict metabolic outcomes.

4.3 Genera analysis - bacteria involved in type 2 diabetes

4.3.1 Genera of bacteria found to be increased in individuals with type 2 diabetes

Lactobacillus, *Escherichia-Shigella*, *Enterococcus*, *Subdoligranulum* and *Fusobacteria* were found to be positively associated with T2DM (Table 4, Figure 3).

4.3.1.1 *Lactobacillus*

The Lactobacillus genus comprises of over 200 physiologically diverse gram-positive, non-spore forming lactic acid bacteria. Despite its positive association with T2DM, *Lactobacillus* species

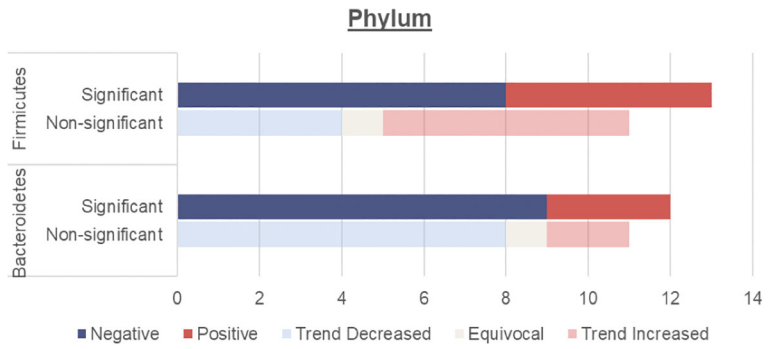

FIGURE 2
Number of human studies reporting Firmicutes and Bacteroidetes abundance and their association with T2DM. Studies were classified as having a significant association with T2DM (either positive or negative) if the p values were <0.05. Studies were classified as having a non-significant association with T2DM if they did not report on p values, had p values >0.05 or an LDA value <4 or >-4. These studies were then further classified into a non-significant association but trend increased, equivocal (equal abundance or not reported), or trend decreased.

such as *Lactobacillus paracasei* (63), *Lactobacillus fermentum* (64) *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* (65, 66) have demonstrated anti-inflammatory properties or benefits on host metabolism as a combination probiotic with *Bifidobacterium lactic* (65, 66).

The positive association of *Lactobacillus* with T2DM may therefore be driven by Metformin. Metformin, a first-line antihyperglycemic agent for treatment of T2DM, may alter bacterial abundances depending on the taxon's resistance or sensitivity to the drug. In 2015, using 784 human gut metagnomes, Forslund et al. confirmed this positive association between metformin and Lactobacillus (55).

Among the eleven studies that reported an increase in *Lactobacillus* abundance (15–17, 21, 33, 49–54), only three studies (21, 50, 52) accounted for metformin use. Among these, one study found higher *Lactobacillus* levels regardless of metformin use (50), one found higher levels only in participants on unspecified oral antihyperglycemic agents (21), while the last study found no difference when accounting for metformin (52). More studies on treatment naïve T2DM or controlled for Metformin use are warranted.

TABLE 4 Genera found to be positively associated with type 2 diabetes.

Genus	Increased	Decreased	No significant difference
Lactobacillus	(15–17, 21, 33, 49–54)	(55)	↑ (12, 25, 26, 36, 47) ↓ (14) Equivocal (19, 41)
Escherichia-Shigella	(12, 13, 16, 18, 38, 40, 47)	(36)	↑ (25, 41, 56) ↓ (55)
Subdoligranulum	(12, 30, 32, 38)	(20, 55)	↑ (26, 36)
Enterococcus	(12, 16, 27)		↑ (26) ↓ (10, 50) Equivocal (51)
Fusobacterium	(16, 26, 34)		↑ (14, 25, 54) ↓ (54, 55)

Studies with no significant differences are reported as trends. ↑ - increase, ↓ - decrease.

4.3.1.2 *Escherichia-Shigella*

The *Escherichia-Shigella* genus, part of the family Enterobacteriaceae, includes multiple opportunistic pathogens (67). These gram-negative bacteria produce proinflammatory components such as lipopolysaccharide (LPS) and peptidoglycans, leading to intestinal and systemic inflammation (12). This systemic inflammation and consequent insulin resistance are key drivers for T2DM.

Unsurprisingly, *Escherichia-Shigella* abundance correlates with variables related to diabetes and obesity, including insulin resistance, diminished beta cell function (56), fasting glucose (41), HBA1c and BMI (47). This genus has been implicated in T2DM complications such as peripheral neuropathy (68), autonomic neuropathy (69), retinopathy (70), diabetic nephropathy (71) and chronic diabetic foot infections (72). *Escherichia-Shigella* has also been associated with an increasing abundance from healthy controls, pre-diabetes to T2DM (56). An increase *in Escherichia-Shigella* has also been associated with metformin use (13, 73). The outlier study that reported decreased *Escherichia-Shigella* abundance may be due to dietary or environmental differences (36).

4.3.1.3 *Subdoligranulum*

Subdoligranulum are anaerobic, spore-free gram-negative bacteria (12). This genera remains relatively underexplored and has only two known species - *Subdoligranulum variabile* and *Subdoligranulum didolesgii*. Four studies (12, 30, 32, 38) found Subdoligranulum more common in T2DM (Table 4) while two studies reported a negative association between T2DM and *Subdoligranulum variabile* (46, 74). These discrepancies may be related to species-specific properties.

Subdoligranulum has been linked to both promotion (75) and reduction of chronic inflammation (74). *Subdoligranulum didolesgii* has been associated with rheumatoid arthritis by triggering synovitis, while *Subdoligranulum variabile* has anti-inflammatory properties through short chain fatty acid (SCFA) production. Decreased levels of *Subdoligranulum variabile* in T2DM individuals may be suggestive of an overall state of inflammation (46).

TABLE 5 Genera found to be negatively associated with type 2 diabetes.

Genus/ Species	Increased	Decreased	No significant difference
Akkermansia	(12)	(34)	↑ (14, 33, 39) ↓ (55) Equivocal (41)
Akkermansia Muciniphila	(32, 57)	(23, 30, 35, 43, 44, 49)	↑ (58)
Bifidobacterium	(12, 15, 16, 18, 26, 59)	(10, 17, 34, 47, 49, 52, 54, 60)	↑ (14, 61) ↓ (25, 39, 50, 55) Equivocal (19, 33, 51)
Bacteroides		(12, 17, 25, 38, 39)	↑ (14, 61) ↓ (26, 47, 55, 59). Equivocal (19, 30, 35).
Roseburia	(10)	(16, 27, 30, 39, 55, 59)	↑ (34, 61) ↓ (12, 14, 17, 18, 26) Equivocal (19)
Faecalibacterium	(10, 16)	(12, 15, 17, 18, 26, 32, 34, 35)	↑ (34, 61) ↓ (14, 39, 40, 47, 55, 59)
Faecalibacterium prausnitzii		(15, 21, 30, 32, 43, 44, 46, 49, 57, 62)	↓ (14, 23, 40) Equivocal (58)
Prevotella	(10, 11)	(15, 16, 36, 38, 51)	↑ (30, 35, 47, 54, 59) ↓ (12, 14, 17, 34, 41, 50, 55, 61) Equivocal (33)

Studies with no significant differences are reported as trends. ↑ - increase, ↓ - decrease.

Subdoligranulum's positive association with T2DM may be influenced by metformin use (55). Of four studies reporting increased *Subdoligranulum*, two did not report metformin use (12, 32), one excluded metformin users (30), and one found an increase regardless of metformin use (38).

4.3.1.4 *Enterococcus*

Enterococcus are gram-positive facultative anaerobic cocci found in intestinal microbiota and on the skin. Some species are opportunistic pathogens causing severe infections such as bacterial endocarditis and spontaneous bacterial peritonitis, while others (*Enterococcus durans*) produce anti-inflammatory SCFAs (76).

Enterococcus may contribute to the development of T2DM through two mechanisms. Firstly, *Enterococcus faecalis* secretes matrix metalloprotease gelatinase causing chronic intestinal inflammation and impaired gut barrier integrity (77), leading to systemic inflammation. Secondly, *Enterococcus* has been linked to impaired glucose homeostasis. Associations include higher HBA1c (16, 27), fasting (27) and post prandial (16) glucose levels, and impaired beta cell function (27). Mechanistically this may relate to overgrowth of enterococcus leading to proportional decreases in beneficial anti-inflammatory bacteria (50).

4.3.1.5 *Fusobacterium*

Fusobacterium are anaerobic gram-negative rod bacteria. Similar to *Enterococcus*, this genus is part of the regular colorectal microbiota. *Fusobacterium*, in particular *Fusobacterium nucleatum*, has been associated with increased production of inflammatory cytokines such as IL-6, IL-8, TNF-α and COX-2 (78). This may contribute to the chronic inflammatory state seen in T2DM. *Fusobacterium* has also been associated with diabetic nephropathy (79) and its species found increased among individuals with T2DM (23, 44).

TABLE 6 Firmicutes-Bacteroides Ratio.

	Increased	Suggestive reduced	Suggestive increased
Firmicutes/ Bacteroides Ratio	(27, 38, 39, 41)	(14, 26, 37, 43)	(33, 48, 58)

Considered suggestive if no significance was reported or if $p > 0.05$.

4.3.2 Genera of bacteria found to be reduced in individuals with type 2 diabetes

Akkermansia, *Bifidobacterium*, *Bacteroides*, *Roseburia*, *Faecalibacteirum* and *Prevotella* were found to be negatively associated with T2DM (Table 5, Figure 4). Species abundance of *Bifidobacterium*, *Bacteroides*, *Roseburia* and *Prevotella* can be found in Supplementary Table 2.

4.3.2.1 *Akkermansia*

Akkermansia is gram-negative bacterium belonging to the Verrucomicrobia phylum. *Akkermansia mucinphilia*, a symbiont microbe colonizing the human intestinal mucosal barrier, is a promising next generation probiotic. It plays a critical role in the maintenance of intestinal barrier, production of anti-inflammatory cytokines and SCFA benefiting host metabolism. In diabetic rat models, administration of live attenuated *Akkermansia* reduced oxidative stress, lipotoxicity, LPS and inflammation (80). In individuals with T2DM, combined probiotics containing *Akkermansia muciniphila* reduced HBA1c and postprandial glucose control (81).

Reduced levels of *Akkermansia mucinphilia* are associated with T2DM. *Akkermansia* is inversely correlated with HBA1c and fasting glucose and positively with anti-oxidants (41).

4.3.2.2 *Bifidobacterium*

Bifidobacterium is a dominant non-spore-forming, gram-positive taxa that help maintain balances between the various intestinal floras (82). Key *Bifidobacterium* species include *Bifidobacteroim bifidum*, *Bifidobacterium adolescentis* and *Bifidobacterium longum*. These species have been used as probiotics in humans (65, 66, 83) and administered in animal studies (84, 85) leading to reduced cytokine production and improved metabolic parameters such as glucose and HBA1c (66, 84).

Apart from SCFA production, *in vivo* and *in vitro* studies show that *Bifidobacterium* administration markedly decreased intestinal permeability by increasing tight junction expression and reducing inflammatory cytokines such as IL-6 and TNF-α (86). This reduces metabolic endotoxaemia, systemic inflammation and may explain its overall negative association with T2DM (Table 5). An increase in

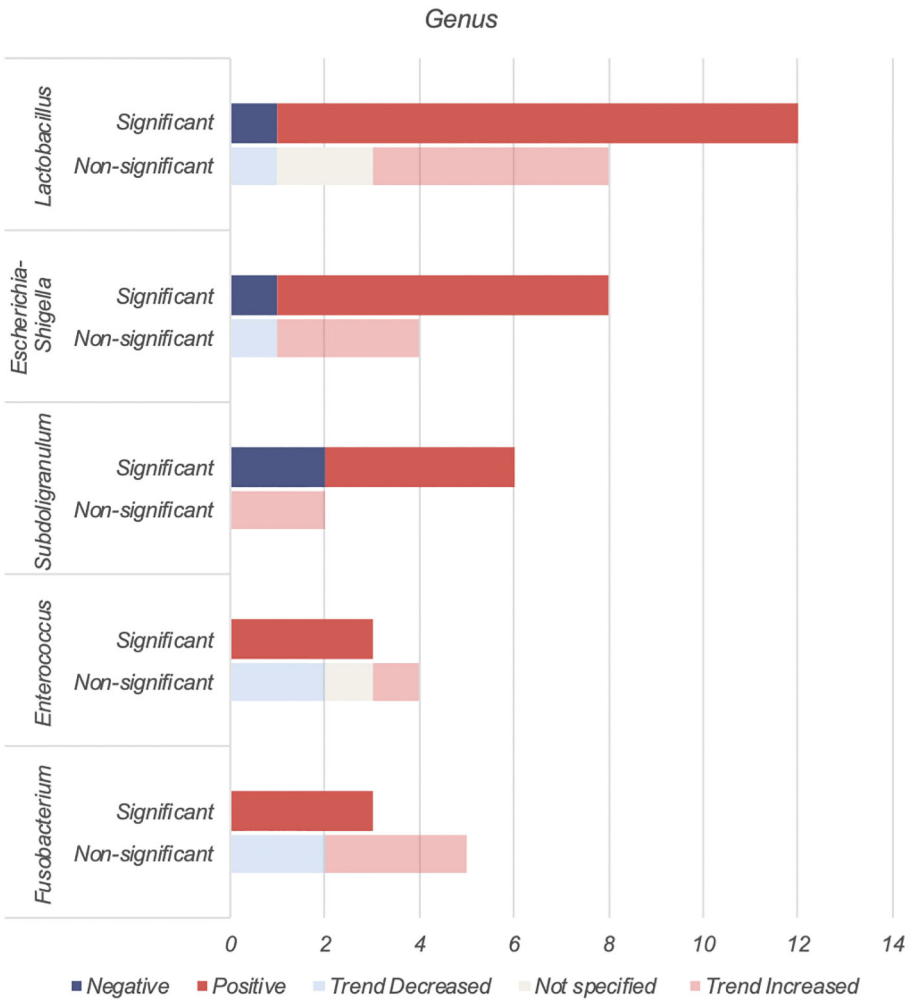

FIGURE 3
Number of human studies reporting on genera found to be positively associated with T2DM.

Bifidobacterium has been attributed to antihyperglycemic agents (16) or a U shaped association with T2DM (26, 59).

4.3.2.3 *Bacteroides*

Bacteroides is a gram-negative obligate anaerobic taxa constituting approximately 25% of the intestinal gut microbiota. As commensals, these taxa generally maintain a beneficial relationship with the human gut. Overall, *Bacteroides* species including *Bacteroides fragilis*, *Bacteroides thetaiotamicron*, *Bacteroides vulgutas* or *Bacteroides dorei* have been associated with a protective effect against T2DM through anti-inflammatory properties (87) and an improved gut barrier integrity from mucus (88) and SCFA production (89). *Bacteroides* species also have a structurally different LPS that is less pro-inflammatory than classical enterobacterial LPS (90). Discrepancies in *Bacteroides* abundance (Table 5) may be due to the bacteriostatic and bactericidal effect of metformin (55) or potential pathogenic Bacteroides species that can contribute to chronic inflammation (39).

4.3.2.4 *Roseburia*

Roseburia is a gram-positive, SCFA-producing member of the Firmicutes phylum that inhabits the human colon. *Roseburia* has been identified as a pathognomonic bacteria in T2DM (91) with significant lower levels in participants. Reduced species include *Roseburia hominis* (23, 46), *Roseburia intestinalis* and *Roseburia inulinivorans* (32, 53, 55). *Roseburia* improves glucose homeostasis and intestinal permeability through SCFA production and anti-inflammatory properties (92). Gut microbiota transplantations from lean donors to recipients with metabolic syndrome led to increased fecal Roseburia and butyrate levels, correlating with improved insulin sensitivity (93).

4.3.2.5 *Faecalibacterium*

Faecalibacterium are human gut colonizers and well-known SCFA producers. *Faecalibacterium* and *Faecalibacterium prausnitzii* were consistently reduced in T2DM (Table 5), with the later being highly discriminant (91). In mice, *Faecalibacterium prausnitzii* administration was associated with improved glucose

levels and HBA1c, making it a promising orally administered probiotic (94). *Faecalibacterium* is negatively associated with HBA1c (39).

4.3.2.6 *Prevotella*

Prevotella has been linked to both pathogenic effects including systemic inflammation and insulin resistance (95) and beneficial effects like SCFA production (96) and reduced gut permeability via increased production of tight junction proteins (97). *Prevotella* is negatively correlated with HBA1c (16, 41, 98), but positively with blood glucose (10, 41). The discrepancies within the *Prevotella* genus may be due to diet (24) and genetic diversity within its species (99).

4.3.3 Genera of bacteria found to have mixed findings in type 2 diabetes

Unlike previous reviews (100), *Blautia* and *Ruminococcus* were found to have mixed associations (Table 7).

4.4 Microbiota effects on metabolism in type 2 diabetes individuals

In T2DM, gut dysbiosis leads to increased systemic inflammation and an unfavorable host metabolism (Figure 5). This is due to an increase in pro-inflammatory cytokine and LPS production, increased gut permeability enabling bacterial endotoxin translocation, and reduced beneficial gut metabolites. Ultimately, systemic inflammation induces insulin resistance and contributes to chronic hyperglycemia and development of complications.

4.4.1 Increased gut permeability

Patients with T2DM have increased intestinal permeability compared to age, sex and BMI matched controls (102). This results in translocation of gut microbes and their products into the bloodstream, in turn causing metabolic endotoxaemia and increased systemic inflammation. This is supported by elevated blood levels of bacterial cell wall products and circulating intestinal bacteria in individuals with pre-diabetes (103) and T2DM (51).

Gut bacterial dysbiosis increases gut permeability via three mechanisms: alterations in expression, canalization and distribution of tight junction proteins; overactivation of the endocannabinoid system; and altered production of beneficial gut metabolites including SCFA and bile acids.

4.4.1.1 Alterations in tight junction proteins

The intestinal lining composed of epithelial cells assisted by tight junctions (TJ), acts as a physical barrier against microorganisms and antigens. TJ controls intestinal permeability (104). In T2DM, reduction in beneficial microbes *Bacteroides*, *Bifidobacterium*, *Faecalibacterium*, *Roseburia* and *Akkermansia*, leads to decreased gene expression and therefore reduced localization, production, and distribution of TJ proteins. This results in increased gut permeability.

Mouse studies show that pre-treatment with *Bifidobacterium* (86), *Bacteroides vulgatus, Bacteroides dorei* (105) or *Prevotella histicola* (97), upregulates TJ genes leading to reduced intestinal permeability and inflammation. *Bacteroides fragilis* (106–108), *Bacteroides facies* (109), *Bifidobacterium bifidum* (110), *Bifidobacterium adolescentis* (111) and *Bifidobacterium longum* (112) have also been found to increase TJ proteins.

Faecalibacterium prausnitzii and *Roseburia intestinalis* reduce gut permeability by production of butyrate and upregulation of TJ proteins (89, 109). Butyrate is essential for colonic epithelial cells, offering anti-inflammatory properties and protecting against pathogens (30). In db/db mice, *Faecalibacterium prausnitzii* also produces microbial anti-inflammatory molecule, increasing TJ expression and restoring the damaged intestinal barrier (113).

Akkermansia muciniphila decreases gut permeability by promoting TJ protein expression via its outer membrane protein Amuc_1100. Additionally, it improves intestinal TJ via AMPK activation in the epithelium (114) and modulation of the endocannabinoid system (115).

Less understood are the bacteria Rumincoccaeceae and *Blautia* which may be associated with increased gut permeability (116). Further studies are needed to confirm these findings and understand their mechanisms.

4.4.1.2 Endocannabinoid system

There is growing evidence that the endocannabinoid system regulates intestinal inflammation and mucosal barrier permeability, thus influencing T2DM pathophysiology.

The endocannabinoid system, historically associated with cognitive and emotional processes, also regulates intestinal inflammation. The two main endocannabinoids are anadamide (AEA) and 2 arachidonylglycerol (2-AG). They act primarily through cannabinoid receptors CB1R and CB2R. CB1R is expressed in gastrointestinal epithelial cells and myenteric and submucosal plexuses while CB2R may be found on enteric neurons (117).

Overactivation of CB1R via AEA and 2-AG leads to increased gut permeability (117). In T2DM mice models, CB1R antagonists were shown to decrease gut permeability by reducing inflammation and alterations in TJ proteins (118). *Akkermansia muciniphila* antagonises CB1R through its outer membrane protein Amuc_1100, reducing gut permeability, LPS levels and systemic inflammation (115). *Bacteroides fragilis* also affects epithelial barrier permeability through the endocannabinoid system (119).

Oxidative stress, inflammation, and insulin secretion contribute to T2DM and its complications. Although unrelated to gut permeability, CB2R activation decreases inflammation and oxidative stress and promotes pancreatic insulin secretion via calcium signal regulation (120). This suggests potential benefits of CB2R agonists in T2DM management.

4.4.2 Alteration to the gut metabolites

The gut microbiota acts as a metabolic organ and facilitates nutrient and energy harvesting from food. It produces metabolites that regulate host metabolism including SCFA and bile acids which

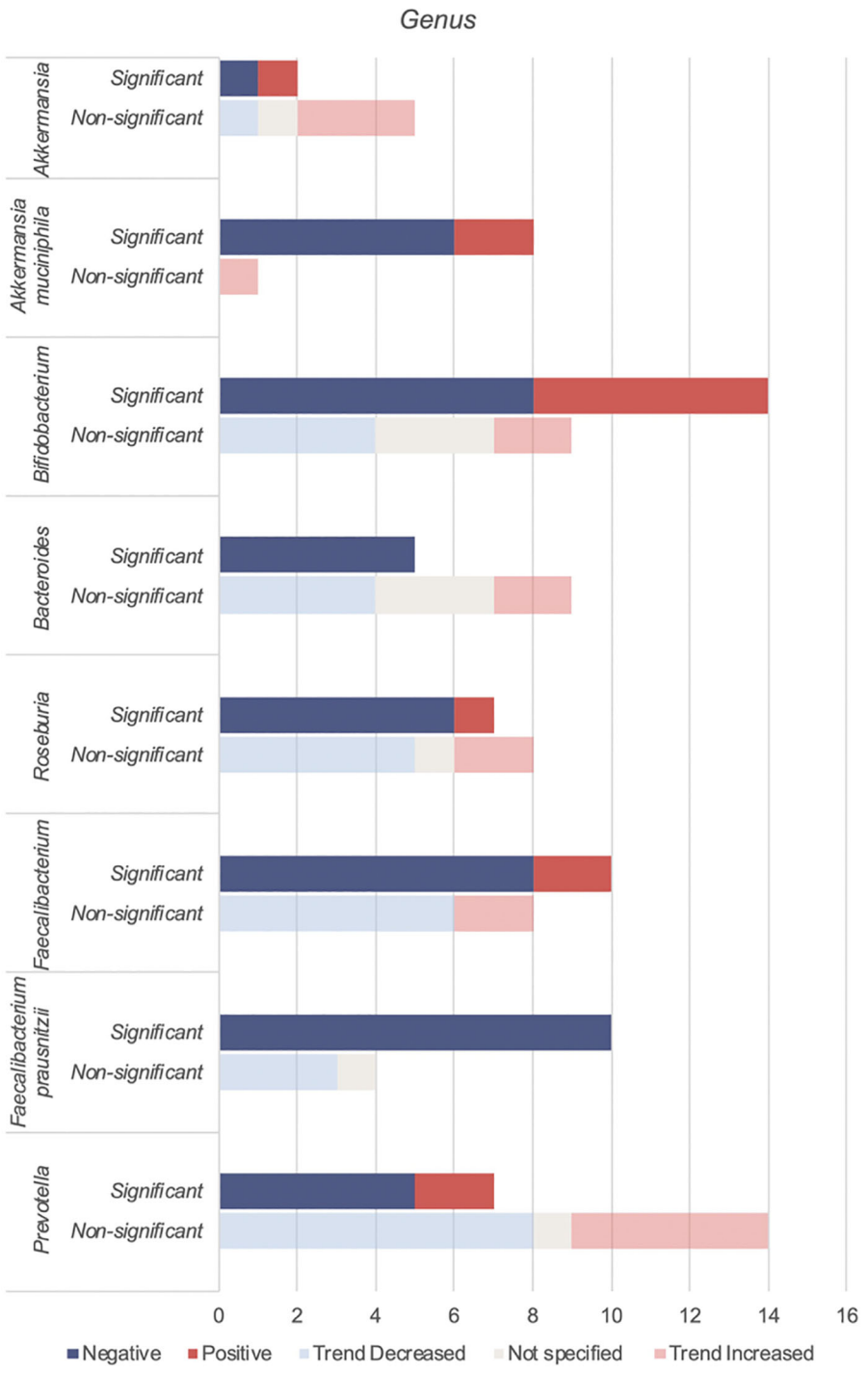

FIGURE 4
Number of studies reporting on genera found to be negatively associated with T2DM.

maintain the intestinal barrier (4). Alterations in the gut microbiota is thus associated with alteration to the gut metabolites which in turn contributes to T2DM and its complications.

4.4.2.1 Alteration to short chain fatty acids

SCFAs are produced by gut microbiota from non-digestible carbohydrates. They provide energy to colonocytes, reduce inflammation and regulate satiety (121). The most common SCFAs are acetate, propionate and butyrate, and are predominantly produced by anaerobic Bacteroidetes and Firmicutes phyla.

SCFAs have multiple beneficial effects such as maintaining gut permeability, modulating host metabolism and anti-inflammatory effects. Reduced levels of SCFA-producing bacteria including *Bacteroides*, *Bifidobacterium*, *Faecalibacterium*, *Prevotella* and

TABLE 7 Genera found to have mixed associations with type 2 diabetes.

Genus	Increased	Decreased	No significant difference
Blautia	(15, 18, 26, 27, 101)	(12, 35, 46, 59, 61)	↑ (38, 39) ↓ (28, 55, 56) Equivocal (30, 41)
Ruminococcus	(17, 30, 39)	(11, 27, 36)	↑ (18, 22, 34, 47) ↓ (10, 12, 14, 28, 38, 40, 55) Equivocal (28, 41, 59, 61)

Studies with no significant differences are reported as trends. ↑ - increase, ↓ - decrease.

Akkermansia. are associated with T2DM. This is reflected by the reduced acetate (38), propinionate (38, 98), butyrate (38, 98) and other SCFA (38, 51) concentrations in T2DM fecal samples. Functional analysis of gut microbiota showed reduced SCFA-producing pathways in T2DM compared to controls (61).

Individuals with T2DM related complications had lower SCFA fecal concentrations than those without complications (38). Increased dysbiosis severity and reduced production of SCFA may contribute to the development and progression of T2DM complications.

4.4.2.1.1 Alteration to SCFA resulting in decreased gut barrier integrity

SCFA help to maintain gut barrier integrity through a number of mechanisms. This includes promoting epithelial growth and innate responses to microbes, providing energy to intestinal epithelial cells via beta-oxidation in the mitochondrial tricarboxylic acid cycle and maintaining an anaerobic gut environment hostile to opportunistic aerobic pathogens (122). SCFA also stabilize transcription factors that protect the barrier and activate genes for TJ proteins thus preventing bacterial and LPS translocation and systemic inflammation (89). Lower SCFA concentrations in T2DM may therefore to altered microbiota diversity and increased intestinal permeability, predisposing to insulin resistance through metabolic endotoxaemia.

4.4.2.1.2 Alteration to SCFA resulting in altered glucose and lipid metabolism

SCFA influence glucose and appetite regulation. In human *in vivo* studies, rectal infusions of SCFA mixtures led to a rise in plasma peptides YY (123–125) and glucagon peptide-1 (GLP-1) (123). This resulted in appetite control, increased insulin sensitivity and increased pancreatic beta cell concentrations (4, 126). SCFA also modulate glucose and lipid metabolism. Propionate suppresses hepatic gluconeogenesis, while acetate and butyrate reduce lipogenesis and increase leptin secretion (122). In mouse models, SCFA increase food intake via parasympathetic activity and support glucose stimulated insulin secretion (127). Reduced levels of SCFA may therefore lead to poor appetite control, hyperglycemia, hyperlipidemia and insulin resistance.

4.4.2.1.3 Alteration to SCFA results in increased inflammation

SCFA exhibit anti-inflammatory properties. Butyrate inhibits NF-kB activation, reducing pro-inflammatory cytokines like TNF-α, IL-6, IL-2, IL-8 and promotes IL-10 production via GPR109A, maintaining a balance between pro and anti-inflammatory T cells (128). Lower SCFA levels may contribute to chronic inflammatory state and insulin resistance in T2DM.

4.4.2.1.4 Alteration to SCFA negatively disrupting the gut environment

Butyrate producing bacteria compete with gram-negative bacteria, maintaining microflora balance and inhibit pathogenic

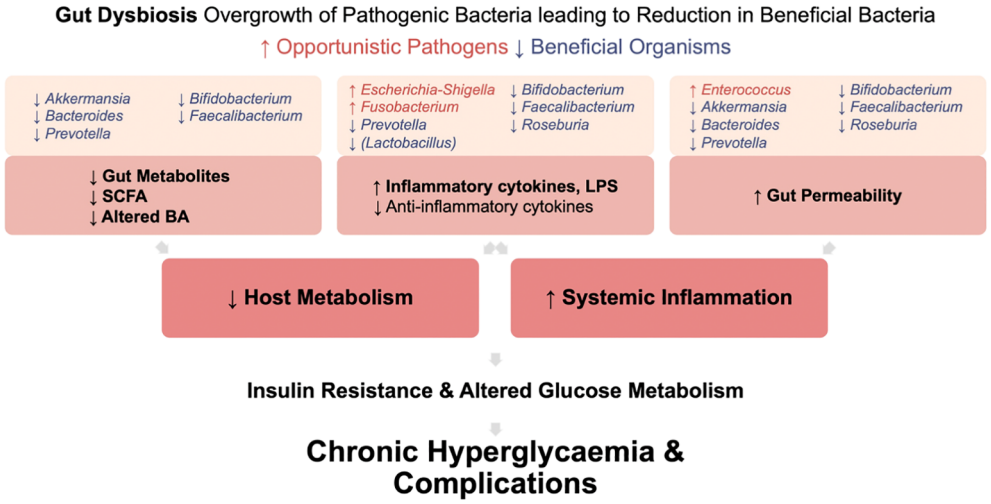

FIGURE 5
Mechanisms by which gut dysbiosis contributes to the development and progression of T2DM. Gut dysbiosis in T2DM leads to increased systemic inflammation and an unfavorable host metabolism. This occurs due to increased production of pro-inflammatory cytokine and LPS, increased gut permeability enabling bacterial endotoxin translocation, and reduced production of beneficial gut metabolites. Ultimately, this systemic inflammation induces insulin resistance. Coupled with altered glucose metabolism in T2DM, these factors contribute to chronic hyperglycemia and the development of complications.

TABLE 8 Abundance of secondary bile acid producing bacteria in type 2 diabetes.

	Increased	Decreased	No significant difference
Ruminococceae		(15, 21, 22, 46)	↑ (11) ↓ (19, 40)
Lachnospiraceae	(18, 32)	(46)	↑ (19) ↓ (11) Equivocal (30)
Clostridium	(32)	(11, 22, 35)	↑ (55) ↓ (10, 14, 47, 61)

Studies with no significant differences are reported as trends. ↑ - increase, ↓ - decrease.

strains. They also maintain an anaerobic environment by enhancing coloncyte oxygen consumption and stabilizing hypoxia inducible factor (122). Depletion of butyrate producing bacteria can lead an increase in opportunistic pathogens like *Fusobacterium*, which releases harmful by-products perpetuating the inflammatory cycle (129).

4.4.2.2 Alteration to bile acids

Bile acids, known for their role in digestion of dietary fats, have recently gained attention due to their possible influence on metabolic processes, particularly in the context of T2DM. Primary bile acids (PBAs), cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from cholesterol in hepatocytes and released into the duodenum. They are then uncoupled by bile saline hydrolysase before being converted into more hydrophobic secondary bile acids (SBAs) through bile acid deconjugation and the rate limiting 7α-dehydroxylase enzyme. *Bacteroides* and *Enterococcus* are involved in the initial deconjugation, while *Bifidobacterium*, *Lactobacillus* and *Enterococcus* utilize bile saline hydrolase. Meanwhile, selected bacteria from the Lachnospiraceae and Ruminococcaceae family perform the subsequent 7α-dehydroxylase conversion of CA and CDCA to generate the SBAs deoxycholic acid (DCA) and lithocholic acid (LCA) respectively (130). The abundance of these bacteria are described in Table 8.

Interestingly, the profiles of bile acids in patients with T2DM vary across different studies. Some studies indicate higher levels of total bile acids, PBA and SBA, among individuals with T2DM (131, 132). In contrast, other studies have found no significant differences in total serum bile acid levels between T2DM patients and controls (133). Nonetheless, the majority of these studies do suggest a relationship between increased insulin resistance and higher total bile acids (132, 133), highlighting the therapeutic potential of targeting bile acids in T2DM. Alterations in bile acids have been associated with complications of T2DM including cardiovascular disease (134) and diabetic kidney disease (135).

4.4.2.2.1 Alteration of bile acids resulting in altered glucose metabolism

Bile acids regulate glucose homeostasis through the Farnesoid X receptor (FXR) and Takeda-G-protein-receptor 5 (TGR5) (136). PBAs preferentially activate FXR, while SBAs favor TGR5. Activation of TGR5 appears to have a beneficial effect on glucose metabolism by stimulating release of GLP-1 from enteroendocrine cells, which enhances insulin secretion, slows gastric emptying and reduces appetite (137). Interestingly, both deactivation and activation of FXR have been linked to positive effects on glycemic regulation. For example, intestinal FXR activation has been associated with reduced hepatic gluconeogenesis (138, 139) and contribute to glucagon fasting-induced hepatic gluconeogenesis (140). FXR deficiency has been linked to increased GLP-1 plasma concentrations (138, 141). Nonetheless, hepatic FXR deficiency in mice has been shown to increase gluconeogenesis, worsening glucose intolerance and insulin resistance (142). This FXR paradox highlights the complexity of FXR signaling, and suggests that the role of FXR in metabolic dysfunction may differ between the liver and intestine (143).

The systematic effects of various secondary bile acids on glycemic control have been demonstrated in both humans and animal models. For example, administration of ursodeoxycholic acid (UDCA) has been shown to improve post-prandial glucose levels and GLP-1 secretion (144), reduce metabolic syndrome (145) and increase the survival rate of pancreatic beta cells (146, 147). Additionally, intrajejunal and rectal taurocholic acid led to decreased blood glucose levels and the release of satiety hormones GLP-1 and Peptide YY (148, 149). Meanwhile, metformin, a drug commonly prescribed for T2DM, has been suggested to modulate primary and secondary bile acid levels and alter the expression of their receptors, thereby enhancing insulin sensitivity (150).

Specifically, among the taxa that differ significantly in individuals with T2DM, *Lactobacillus* and *Bifidobacterium* have been suggested to play a role in modulating bile acids and improving glycemic control. In a recent randomized control trial, a probiotic product containing *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Bifidobacterium animalis subsp. lactis M8* and *Bifidobacterium animalis subsp. lactis V9.* led to reductions in HbA1c and fasting blood glucose levels, along with increased insulin secretion. Faecal metabolite analysis demonstrated an increase in both CDCA and hyodeoxycholic, a component of hyoholic acid shown to upregulate GLP-1 secretion via TGR5 (139). The study suggested that specific bile acids may activate various receptors, which in turn promotes GLP-1 secretion, thereby reducing blood glucose levels (151). Collectively, these findings highlight the potential therapeutic value of bile acids in T2DM.

4.4.2.2.2 Alteration to bile acids affecting gut barrier integrity

Alterations in bile acid profiles affect intestinal permeability through regulation of TJ proteins. In murine models, DCA reduces TJ protein Zona-Occludens-1, thereby increasing gut permeability (152). Primary biliary acids CDCA and CA, and secondary biliary acids DCA, increase epithelial permeability through phosphorylation of occludin in intestinal Caco cells (153). At high concentrations DCA is cytotoxic to intestinal stem cells and goblet cells, thereby impairing gut permeability (154). Conversely, LCA reduces intestinal permeability by ameliorating TNF-α induced disruption of TJ proteins (155). In murine models, an increase in LCA and DCA was associated with increased colon expression of TGR5 and TJ proteins, thereby improving gut-barrier integrity (156). Human studies demonstrate that elevated levels of

LCA and DCA have anti-inflammatory properties within the colon (157). Bile acids have both beneficial and detrimental effects on intestinal permeability, and further studies are required to understand their specific impacts.

4.4.2.2.3 Alteration in bile acids resulting in systemic inflammation

Bile acids have been shown to inhibit the induction of pro-inflammatory genes and the production of inflammatory cytokines by macrophages via FXR and TGFR-5 receptors (158). In mice models, the production of secondary bile acids, such as LCA and UDCA, ameliorated colitis and reduced the production of proinflammatory cytokines TNF- α, IL-17A and IL-6 (156). Alteration in bile acids can thus lead to decreased anti-inflammatory effects and contribute as well as exacerbate the chronic low-grade inflammatory state in T2DM.

In summary, bile acids play a role in modulating intestinal permeability, systemic inflammation, and glucose homeostasis, thereby contributing to the pathogenesis of T2DM. While bile acids represent a promising therapeutic target, the precise abundance of various bile acids in T2DM and their effects on different receptors, particularly FXR, remain unclear. Further studies are needed to confirm these alterations and clarify the specific interactions involved.

4.4.2.3 Increased systemic inflammation

T2DM is associated with chronic low-grade systemic inflammation caused by metabolic endotoxaemia and cytokine stimulation by microbes leading to oxidative stress, macrophage activity and insulin resistance. Insulin resistance occurs due to activation of the inflammatory cascade, subsequent activation of serine kinases, insulin receptor substrate serine phosphorylation and consequent insulin signaling inhibition causing cellular insulin resistance (159).

4.4.2.3.1 Metabolic endotoxaemia

In T2DM, metabolic endoxaemia occurs due to increased production of toxic bacterial components and increased gut permeability enabling translocation of these products into the systemic circulation.

4.4.2.3.1.1 *Lipopolysaccharide*

Gram-negative bacteria, such as *Fusobacterium* and *Escherichia-Shigella*, produce LPS an endotoxin that activates immune responses by binding to pattern recognition receptors such as toll-like receptor 4 (TLR4), NLRP3 inflammasome and NOD-like receptors which are expressed on the surfaces of antigen presenting cells. This leads to release of pro-inflammatory cytokines IL-1, IL-7, TNF-α release (121) and insulin resistance via inhibition of insulin signaling (159). Gut dysbiosis in T2DM increases LPS synthesis (46, 57) with higher plasma levels of LPS (15, 51) and TLR4 receptor activation (15) observed.

4.4.2.3.1.2 *Decreased intestinal alkaline phosphatase due to gut dysbiosis contributes to metabolic endotoxaemia in T2DM*

IAP is an enzyme which mitigates intestinal inflammation through detoxification of pathogen toxins and regulation of gut microbes (160). It de-phosphorylates LPS, reducing its toxicity and lowering systemic inflammation (161). In mice, IAP was shown to reverse metabolic endotoxaemia (162). Very low levels of fecal IAP have been reported in T2DM patients (163). *Bifidobacterium* species, *Faecalibacterium prausnitzii, Roseburia* species and other butyrate producing bacteria modulate IAP activity (164). A decrease in these anti-inflammatory, butyrate producing bacteria may contribute to chronic systemic inflammation in T2DM.

4.4.2.3.2 Cytokine modulation

T2DM is associated with elevated pro-inflammatory cytokines. Bacterial taxa such as *Escherichia-Shigella* and *Fusobacterium* are increased in T2DM and correlate with higher levels of pro-inflammatory cytokines like IL-17, TNF-α and IL-6 (165).

Conversely, beneficial microbes *Roseburia intestinalis* (166), *Prevotella histicola*(97), *Faecalibacterium prausnitzii* (167), *Bifidobacterium longum* (167), *Bacteroides fragilis* (87, 168), *Akkermansia muciniphila*(169), *Lactobacillus paracasei* (63) and *Lactobacillus fermentum* (64) promote anti-inflammatory cytokine IL-10 production and suppress pro-inflammatory cytokines (87, 92, 166, 167, 170, 171). Butyrate producing bacteria such as *Roseburia*, *Faecalibacterium* and *Subdoligranulum* also decreases pro-inflammatory cytokine production by inhibiting NF-kB, a major transcription factor essential for inflammatory responses (128).

4.4.2.4 Preferential growth of pathogenic microbiota

Pathogenic bacteria including *Enterococcus* and *Escherichia-Shigella* may outcompete beneficial bacteria, such as *Faecalibacterium*, *Roseburia* and *Bifidobacterium*, perpetuating negative effects on gut health and inflammation.

5 Limitations

This systematic review has several limitations. The significant variation in methodology across various human observational studies made it difficult to draw definitive conclusions. Differences in inclusion and exclusion criteria, and varied methods for controlling factors such as age, BMI, diet and medication, affected bacterial abundances and hindered efforts for consistent comparisons. Furthermore, few studies provided raw data on bacterial abundances or reported non-significant bacterial abundances, complicating quantitative data pooling for any specific bacteria.

Most studies did not account for the effects of metformin and other oral anti-hyperglycemic agents, which are known to alter certain bacterial abundances. This review could not control for their use, highlighting the need for future large-scale studies to at least account for, if not control, the effects of these diabetes medications.

Majority of the studies utilized 16s RNA gene sequencing, with few studies utilizing metagenomic sequencing. This meant that it was rare to identify microbes at species or strain levels and may account for some discrepancies at the genus level. Moreover, few studies examined functional alterations in T2DM and correlated it to individual bacterial taxa. Therefore, only associations but not

causations between taxa and T2DM could be determined. Future research should assess the functional potential of the gut microbiome in individuals with T2DM.

Finally, the pathogenesis, perpetuation and management of T2DM is multifactorial and various clinical factors including genetics, other comorbidities, adherence to therapies and presence of complications all play a critical role. Future studies should measure these factors, and consider their interplay with gut microbiota in T2DM.

6 Conclusion

This systematic review demonstrates that T2DM is strongly associated with gut dysbiosis, as evidenced by differential microbial abundances, altered F/B ratio and changed diversity indices. Through increased gut permeability, decreased SCFA production and modulation of inflammatory cytokines, gut dysbiosis leads to increased systemic inflammation and disrupted glucose homeostasis.

Among these microbes, *Escherichia-Shigella* is consistently associated with T2DM, while *Faecalibacterium*, in particular *Faecalibacterium prausnitzii* appears to offer a protective effect against T2DM. However, the heterogenicity and observational nature of these studies hinder establishment of causative relationships. Future research should control for factors such as age, diet and medication use, and incorporate functional analysis of these gut microbes.

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

SC: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. ML: Methodology, Writing – review & editing. DC: Methodology, Writing – review & editing. SJ: Conceptualization, Formal analysis, Project administration, Supervision, Writing – review & editing. NL: Conceptualization, Formal analysis, Investigation, Methodology, 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1486793/full#supplementary-material

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Protein-bound uremic toxins as therapeutic targets for cardiovascular, kidney, and metabolic disorders

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Cardiovascular-kidney-metabolic (CKM) syndrome is a systemic clinical condition characterized by pathological and physiological interactions among metabolic abnormalities, chronic kidney disease, and cardiovascular diseases, leading to multi-organ dysfunction and a higher incidence of cardiovascular endpoints. Traditional approaches to managing CKM syndrome risk are inadequate in these patients, necessitating strategies targeting specific CKM syndrome risk factors. Increasing evidence suggests that addressing uremic toxins and/or pathways induced by uremic toxins may reduce CKM syndrome risk and treat the disease. This review explores the interactions among heart, kidney, and metabolic pathways in the context of uremic toxins and underscores the significant role of uremic toxins as potential therapeutic targets in the pathophysiology of these diseases. Strategies aimed at regulating these uremic toxins offer potential avenues for reversing and managing CKM syndrome, providing new insights for its clinical diagnosis and treatment.

KEYWORDS

protein-bound uremic toxins, gut microbiota, cardiovascular-kidney-metabolic syndrome, metabolic diseases, management

Background

In recent years, chronic kidney disease (CKD) has become a global epidemic, with a prevalence rate as high as 14.3% (1). The incidence of CKD is increasing due to rising rates of diabetes, hypertension, and obesity. In China, the adult prevalence of CKD is 10.6% (2). Its complex pathogenesis and lack of effective interventions lead to multisystem complications (3), making it a significant global public health concern. CKD-related diseases caused approximately 1.2 million deaths worldwide in 2017, with this number projected to increase to 2.2 million by 2040 (4). Cardiovascular disease (CVD) is the most common complication and leading cause of death associated with CKD (5). Metabolic,

cardiovascular, and kidney diseases significantly overlap, presenting major health challenges with high morbidity and mortality rates. These conditions often coexist and interact, underscoring the interconnectedness of heart, kidney, and metabolic health. In 2023, the American Heart Association (AHA) proposed a new disease concept—Cardiovascular-Kidney-Metabolic (CKM) syndrome, emphasizing the pathophysiological interactions among metabolic risk factors, CKD, and CVD. This syndrome is characterized by systemic diseases leading to multi-organ dysfunction and increased cardiovascular adverse events (6).

A long-term retrospective study in Sweden, involving over a million patients with diabetes mellitus (DM) and control subjects, with a median follow-up of 7.5 years, observed an increasing trend in end-stage renal disease (ESRD) among DM patients compared to controls. The incidence rate of ESRD (per 100,000 person-years) was 116.1 in patients with DM versus 42.0 in the control group. Further analysis revealed associations between cardiac metabolic risk factors in patients with DM and increased risk of ESRD, including elevated glycated hemoglobin, systolic blood pressure, body mass index, advanced age, and low high-density lipoprotein cholesterol levels (7).Studies have shown that 93.6% of patients with type 2 diabetes mellitus (T2DM) have at least one concurrent cardiovascular-kidney-metabolic disease, with 51% having three or more (8). Epidemiological research and clinical trial data indicate that successfully controlling multiple CVD risk factors can reduce the risk of CVD events by ≥50% (9).

Increasing evidence suggests that the progression of metabolic, cardiovascular, and kidney diseases is associated with the accumulation of uremic toxins, particularly in CKM syndrome stages II-IV (10). Elevated levels of indoxyl sulfate (IS), were found in urine samples from patients with DM and correlated with changes in proteinuria (11, 12). A 5-year follow-up study involving 521 patients with CKD confirmed that impaired kidney function and increased uremic toxins accelerate atherosclerosis. Higher plasma levels of colonic uremic toxins such as trimethylamine-N-oxide (TMAO) in CKD subjects were associated with a 2.8-fold increased risk of CVD-related mortality, and worsened overall survival with increasing TMAO levels (13). In animal experiments, administering a TMAO inhibitor to mice on a high-choline diet reduced plasma TMAO levels and foam cell formation, thereby improving atherosclerotic plaque formation, suggesting a potential therapeutic approach for treating cardiac metabolic diseases (14, 15). Clinical management of CKM syndrome currently focuses on stage-specific drug selection and multidisciplinary approaches. Although conventional treatments, such as lifestyle interventions, glycemic control, and pharmacotherapy, can delay the progression of CKM syndrome, the incidence of adverse heart-kidney outcomes remains high. Patients with both DM and kidney disease have an all-cause mortality rate approximately 30 times higher than those with diabetes alone (16). Even with comprehensive treatment strategies, disease progression is not fully prevented, with a 41.6% mortality rate over a 14-year follow-up (17). Large-scale cohort studies have confirmed that baseline CKD, CVD, and cardiac-kidney comorbidity significantly increase the risk of all-cause mortality by 1.5-fold, 1.8-fold, and 2.4-fold, respectively, compared to those without these conditions. Thus, there is an urgent need to explore therapeutic strategies that reduce the incidence and mortality of CKM syndrome. Uremic toxins such as IS and TMAO have emerged as specific risk factors. Strategies aimed at regulating these uremic toxins offer potential avenues for reversing and managing CKM syndrome, providing new insights for its clinical diagnosis and treatment.

Unique environment of protein-bound uremic toxins in CKM syndrome

Production and metabolism of gut-derived uremic toxins

Currently, over 100 uremic toxins have been identified in the serum of patients with CKD, including a type known for its high protein affinity, called protein-bound uremic toxins (PBUTs). PBUTs, such as IS, p-cresyl sulfate (PCS), and TMAO, primarily originate from endogenous metabolic products, intestinal bacterial breakdown, and exogenous intake. As renal failure progresses, dysbiosis of the gut microbiota, changes in enzymatic activity, and ineffective renal excretion lead to the accumulation of PBUTs, exacerbating multi-organ damage, including to the heart and kidneys (18–20).

Many uremic toxins derive from dietary components such as tryptophan, tyrosine, and choline metabolism. Gut bacteria metabolize dietary tryptophan into uremic toxin precursors, which pass through the intestinal mucosal barrier via the portal vein to the liver. There, they couple with sulfate ions to transform into PBUTs, IS (21). Tyrosine and phenylalanine metabolites generate p-cresol, which is absorbed by the intestines and converted to PCS through the action of sulfotransferases in intestinal epithelial cells, with a small portion metabolized in the liver into glucuronide-conjugated p-cresol (22). Phenylacetic acid in host liver cells forms phenylacetylglutamine (PAGln) (23). Trimethylamine (TMA) is absorbed and circulates to the liver, where it is converted to TMAO (24). Ultimately, uremic toxin precursors are transformed into PBUTs and secreted via renal tubules into urine (25).

In renal tissue, organic anion transporters (OATs) on the basolateral side of proximal tubule cells play a crucial role in the selective absorption of PBUTs from the blood and their active secretion into the renal tubular lumen (26, 27). In patients with CKD, the abnormal increase in uremic toxins primarily stems from changes in intestinal microbiota, leading to increased toxin precursors and reduced secretion through renal tubules due to declining kidney function. In the context of PBUTs accumulation in the bloodstream, increased OATs expression stimulated by CKD leads to significant PBUTs secretion. However, further renal tubular toxicity and fibrosis worsen renal function, failing to compensate for toxin accumulation. Accumulated toxins can specifically bind to the aryl hydrocarbon receptor (AhR), participating in systemic intracellular signaling pathways related to uremic toxicity, and contributing to the occurrence of CKD and its complications, such as CVD (28) (Figure 1).

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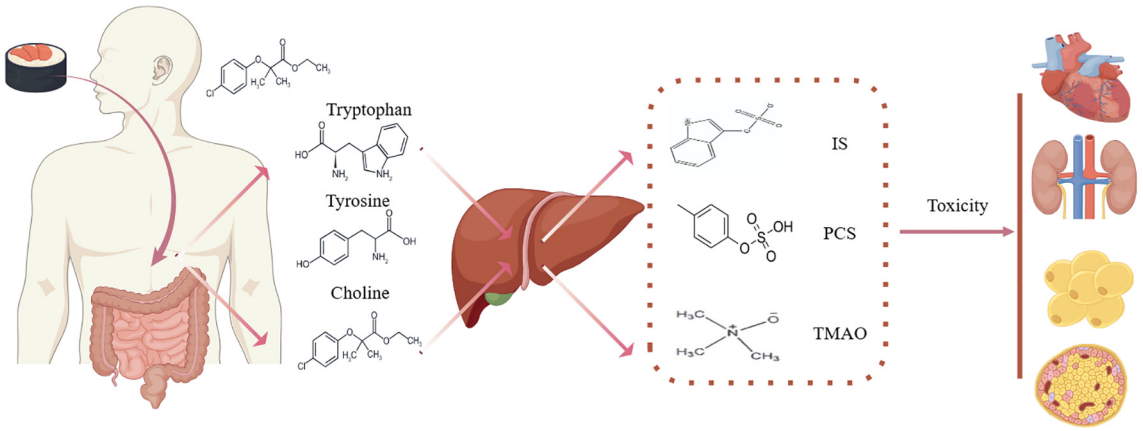

FIGURE 1
Protein-bound uremic toxins in CKM syndrome. IS, indoxyl sulfate; PCS, p-cresol; TMAO, trimethylamine-N-oxide.

Interaction between PBUTs and CKD

According to the "gut-kidney axis" hypothesis, patients with CKD experience a disruption of the "healthy" microbial community in the gut. Gut microbiota produce large quantities of PBUTs, such as IS, PCS, and TMAO, which contribute to kidney damage. Increased gut barrier permeability allows these PBUTs to enter the bloodstream. Toxins produced by harmful bacteria accumulate in the blood, causing persistent inflammation, oxidative stress, immune responses, and alterations in microbial metabolism and composition. This forms a vicious cycle, leading to the development and progression of CKD and its complications (29, 30). In a large-scale clinical cohort study involving 223 patients with ESRD and 69 gender- and age-matched healthy controls, metabolomics analysis indicated that gut microbiota in patients with ESRD caused metabolic alterations characterized by the accumulation of several PBUTs and secondary bile acids (31). Furthermore, our team conducted deep metagenomic sequencing on different stages of kidney disease compared to healthy individuals, identifying 54 high-quality microbial genome-assembled genomes with differential presence. Functional analysis revealed more genes encoding PBUTs, antibiotic resistance, and virulence factors in functional groups positively correlated with disease severity, suggesting a role of PBUTs in the occurrence and progression of kidney disease (32).

Accumulated PBUTs in patients with CKD inhibit the expression of genes associated with tight junction proteins such as zonula occludens-1 and claudins, leading to intestinal barrier damage and increased permeability (33–35). This allows PBUTs to enter the bloodstream and damage the kidneys. IS and PCS can increase reactive oxygen species (ROS) content and triphosphopyridine nucleotide (NADPH) oxidase activity in a time-dependent manner, significantly raising downstream mRNA expression of transforming growth factor-β (TGF-β1) and tissue inhibitor of metalloprotease-1 (TIMP-1), thereby damaging renal tubular cells (36, 37). Additionally, IS and PCS activate the local renin-angiotensin system in the kidneys, increasing levels of TGF-β1, promoting sustained renal hyperperfusion and fibrosis (38–40). Various antigens produced by potential pathogens in the gut can activate immunity, triggering a cascade of inflammatory reactions, leading to a unique state of "chronic inflammatory immune suppression" that induces glomerulosclerosis and tubulointerstitial fibrosis (41–43). Renal biopsy results show significant aggregation of M1 macrophages in early CKD stages, transitioning to M2 macrophages in later stages, promoting renal damage repair and fibrosis (44–46). In CKD mouse models, antibiotic treatment can reduce M1 and M2 polarization in bone marrow-derived macrophages induced by TMAO, thus alleviating renal fibrosis progression (47).Studies indicate that PCS interferes with antigen presentation by macrophages and inhibits T helper cell 1immune responses, leading to adaptive immune dysfunction in patients with CKD (48). PCS also directly inhibits macrophage immune responses and reduces peripheral blood B cell counts (29).

In addition to causing common oxidative stress, inflammation, and immune effects that contribute to renal damage in patients with CKD, PBUTs can also alter autophagy and epigenetic states, furthering CKD progression. A clinical study found almost non-existent mRNA levels of autophagy-related genes in patients with ESRD, indicating impaired autophagy activation in patients with CKD (49). DNA methylation, an important epigenetic regulatory mechanism, requires methyl donors derived from choline metabolized by gut microbiota. In the absence of gut microbiota, intestinal DNA methylation levels significantly decrease (50–52).

Cardiovascular effects of PBUTs

An increasing number of studies have linked the aforementioned PBUTs with cardiovascular mortality in patients with CKD. Compared to non-CKD patients, those with CKD exhibit a twofold higher CVD mortality rate, with the risk increasing with the severity of CKD (53). In a study involving 147 patients with CKD, elevated plasma IS levels were associated with major adverse cardiovascular events, independent of glomerular filtration rate (GFR) and nutritional status (54). Clinically, elevated plasma TMAO levels have been established as an independent predictor of cardiovascular risk and validated in a large

prospective cohort study (55). Two meta-analyses indicated that higher TMAO levels are associated with a 23%-67% increased risk of CVD events and a 55%-91% increased risk of all-cause mortality (56, 57). Additionally, in a cohort of 4000 coronary artery disease patients undergoing selective diagnostic cardiac assessment, those with higher plasma levels of PAGln, especially patients with concomitant T2DM, experienced higher rates of major adverse cardiac events over three years (23, 58).

The impact of PBUTs on cardiovascular pathology primarily manifests as arteriosclerosis, thrombosis, vascular calcification, neointimal hyperplasia, and myocardial fibrosis, ultimately leading to conditions such as myocardial infarction, heart failure, arrhythmias, stroke, and peripheral artery disease. TMAO promotes thrombosis and exacerbates arteriosclerosis through mechanisms involving macrophage scavenger receptors, foam cell activation, endothelial cell activation, increased platelet reactivity, and inhibition of reverse cholesterol transport (59, 60). Additionally, TMAO induces tissue factor and vascular cell adhesion molecule-1 expression in human microvascular endothelial cells, significantly enhances Ca^{2+} stimulation and platelet aggregation, influences vascular calcification, and promotes the progression of arteriosclerosis (61). Long-term dietary supplementation of carnitine in mice alters gut microbiota composition, significantly increasing the synthesis of TMA and TMAO, which aggravates arteriosclerosis. However, inhibiting gut microbiota prevents this phenomenon (62). Thus, modifying gut microbiota to reduce the production of protein-bound uremic toxins can improve cardiovascular outcomes.

IS, a classic PBUT, enhances platelet activity, increases reactions involving collagen and thrombin, and elevates microparticles and platelet-monocyte aggregates derived from platelets. IS binds to the ligand-binding domain of AhR in endothelial cells and vascular smooth muscle cells, activating AhR and inducing tissue factor transcription (63, 64). Concurrently, IS induces ROS expression and stimulates the expression of inflammatory cytokine, mediating thrombosis, vascular injury, and myocardial fibrosis (65, 66).

Interaction between PBUTs and metabolic syndrome

A meta-analysis of 25 cohort studies, 3 cross-sectional studies, and 19 case-control studies confirmed that obesity increases the risk of developing CKD in the general population (67). Elsa et al. (68) demonstrated that overweight and obesity in middle-aged individuals increase the risk of CVD by 31% and 76%, respectively, while in older adults, the risks are elevated by 22% and 40%, respectively. Additionally, approximately 20%-40% of diabetic patients also have kidney disease (69), with CVD being a major cause of morbidity and mortality in patients with T2DM (9). In a large retrospective study involving over ten thousand participants with an average age of 41.8 years and a follow-up period of 3.7 years, the presence of metabolic syndrome was analyzed for its association with all-cause mortality and CVD mortality risk. The results indicated that metabolic syndrome increases the risk of all-cause mortality in women and CVD mortality risk across the entire population (70). This increased risk of metabolic disorders is primarily caused by insulin resistance and obesity, ultimately leading to chronic dysfunction of the heart and kidneys, highlighting the need for early intervention and aggressive treatment upon diagnosis of metabolic syndrome.

PBUTs and obesity

An increasing number of studies indicate that disruptions in gut microbiota and the accumulation of PBUTs play significant roles in the development of obesity. For instance, individuals with severe obesity exhibit higher proportions of *Firmicutes* and *Proteobacteria* compared to healthy obese and lean individuals. These bacteria can metabolize dietary components into precursors of uremic toxins such as phenylacetic acid (PAA) and TMA (71, 72). A meta-analysis exploring the association between circulating TMAO levels and obesity risk reveals a positive correlation between TMAO and increased BMI (73). Furthermore, a dose-dependent relationship between TMAO and obesity has been observed even in seemingly healthy individuals. Using 16S rRNA sequencing and untargeted metabolomics to analyze differences in gut microbiota, plasma, and intestinal metabolism between rats fed a high-fat diet and those on a normal diet, it was found that changes in gut microbiota included decreased abundance at the phylum level and reduced levels of *Akkermansia*, *Ralstonia*, *Bacteroides*, and *Faecalibacterium* at the genus level; Significant alterations were also observed in intestinal and plasma metabolite levels (74). Previous studies have demonstrated that Sangzhi alkaloids (SZ-A) alleviate high-fat diet-induced obesity, improve fat tissue metabolism, and reduce inflammation associated with obesity (75). Oral administration of SZ-A significantly reduces body weight, fat mass, total cholesterol, and low-density lipoprotein levels in high-fat diet-induced obese mice. Interestingly, SZ-A also modulates gut microbiota and alters fecal metabolite composition in obese mice. Compared to the high-fat diet group, SZ-A improves the proportions of *Firmicutes* and *Proteobacteria* at the phylum level and significantly increases the abundance of *Bacteroidetes* and *Akkermansia muciniphila* at the genus level. This change affects the relative abundance of microbial genes involved in PBUTs metabolism. Overall, SZ-A alleviates obesity and metabolic syndrome in high-fat diet-induced obese mice by improving gut microbiota and their metabolic characteristics (76).

PBUTs and diabetes

Research has confirmed a link between alterations in gut microbiota and the accumulation of PBUTs with host insulin sensitivity, glucose metabolism, and impaired amino acid metabolism related to DM. Our team previously identified changes in gut microbiota in patients with DM (77). In a case-control study involving thousands of newly diagnosed T2DM cases and controls, plasma TMAO concentrations were measured and found to be elevated in patients with DM (78). Within patients with DM, gut microbiota convert tryptophan into indole and its derivatives, which act on the AhR pathway, leading to reduced production of glucagon-like peptide-1 (GLP-1) and Interleukin-22. This contributes to increased intestinal permeability and translocation of lipopolysaccharides, resulting in inflammation, insulin resistance, and hepatic steatosis (79). Screening of 130

patients with T2DM revealed that IS levels increase with urinary proteinuria, and PCS also showed an upward trend in urine (80). Treatment of non-DM and early-stage hyperglycemic diabetic mice with a sodium-glucose co-transporter-2 inhibitor for one week not only lowered blood glucose levels but also reduced the formation of PBUTs such as IS by gut microbiota, thereby decreasing their systemic exposure and the need for renal detoxification. This provides a metabolic basis for kidney and cardiovascular protection (81, 82). Therefore, as blood glucose levels rise in patients with DM, exacerbating renal excretory burden, accumulated PBUTs play a "bridging" role, manifesting as a triangular relationship among "diabetes, gut microbiota/PBUTs, diabetic complications," demonstrating interactions, mutual influences, and mutual development among these three entities.

Nutritional intervention

Low-protein diet

Dietary intervention has long been the cornerstone of treatment for patients with CKM syndrome, aimed at reducing the intake of precursors to PBUTs. This approach addresses the root cause to diminish PBUTs production, thereby preventing the onset and delaying the progression of CKM syndrome.

The 2022 edition of *the CKD Early Screening, Diagnosis, and Treatment Guidelines* (82) specifies protein intake recommendations for both non-diabetic CKD and diabetic CKD patients, emphasizing a reduction in overall protein intake while ensuring adequate high-quality protein intake. An experimental evaluation of a low-protein diet (LPD) among CKD patients demonstrated significant reductions in serum levels of IS and PCS in those adhering to LPD (83). Additionally, the PREDICT 1 study from the UK, involving thousands of participants, correlated gut microbiota composition with habitual diets and cardiovascular metabolic markers in blood. The research identified specific components of the microbiota associated with dietary intake and multiple measures of cardiovascular metabolic health, suggesting the potential use of gut microbiota as biomarkers for cardiovascular metabolic risk and strategies to reshape the microbiota to improve personalized dietary health (84). Among healthy species, *Firmicutes* showed the highest correlation; while *Clostridium* difficile was associated with overall poor health. These gut microbiota all participated in the metabolic processes of PBUTs. In addition to a LPD with adequate high-quality protein, CKM syndrome patients, especially those in stages II-IV of CKD, often suffer from complications such as calcium and phosphorus metabolism disorders, hyperkalemia, and hypertension. Therefore, they also need to adhere to low-phosphorus, low-potassium, and low-sodium diets.

Special dietary patterns

Mediterranean diet

The Mediterranean diet (MD) is characterized by high consumption of vegetables, legumes, fruits, nuts, whole grains, and dairy, abundant use of extra virgin olive oil, and encourages the selection of lean proteins (85). It has protective effects against CKD syndrome, obesity, diabetes, chronic kidney disease, and cardiovascular diseases (86–89). A meta-analysis of 70 studies analyzed the correlation between adherence to the MD and the risk of major chronic diseases (T2DM, CKD, and CVD), consistently demonstrating a significant negative association between higher adherence to the MD and the risk of these chronic diseases (86). Adherence to the MD pattern is associated with distinct characteristics of the gut microbiota.

An evaluation of the gut microbiota and metabolome of 153 individuals with different dietary habits, stratified by diet type and adherence to the MD, revealed a significant correlation between vegetable-based diets and increased levels of fecal short-chain fatty acids(SCFAs), *Prevotella*, and certain fiber-degrading *Firmicutes*. Conversely, higher urinary TMAO levels were detected in individuals with lower adherence to the MD (90). An 8-week isocaloric MD dietary intervention study (n=82) showed changes in various microbial features in the gut, including increased abundance of major dietary fiber metabolites, decreased PBUTs metabolites, and beneficial changes in cardiovascular metabolic biomarkers (90).

Additionally, a parallel randomized controlled trial involving 82 healthy overweight and obese participants found significant reductions in plasma cholesterol in the MD group compared to the control group consuming a regular diet. Metagenomic analysis indicated changes in the gut microbiota reflecting increased gene richness in participants with reduced systemic inflammation and PBUTs during the intervention. Higher levels of microbial carbohydrate degradation genes associated with fiber degradation and butyrate metabolism were also observed (91).

Intermittent fasting

Clinical trials on overweight adults have shown that intermittent fasting is beneficial in various contexts such as obesity, diabetes, and cardiovascular diseases (92, 93). Intermittent fasting is increasingly recognized as a promising approach to managing CKM syndrome, potentially improving lifestyle and cardiovascular metabolism to prevent the onset of T2DM and CVD. Su et al. (94) evaluated the impact of intermittent fasting on gut microbiota in young and middle-aged healthy non-obese individuals, finding significant reshaping of the gut microbiota with increased *Clostridiaceae*, *Helicobacteraceae*, and butyrate-producing bacteria, and decreased *Prevotellaceae*, which produce TMAO. These changes contributed positively to blood sugar levels, weight, and body fat. However, a follow-up study involving thousands of diabetic patients found a linear inverse correlation between daily eating frequency and overall mortality as well as cardiovascular disease-related mortality, with HRs (95% CIs) of 0.88 (0.80-0.98) and 0.77 (0.63-0.93), respectively (95). Another large-scale follow-up experiment over 8.0 years found that intermittent fasting was significantly associated with increased cardiovascular mortality risk (95). Currently, there is no consistent conclusion on whether intermittent fasting is beneficial

in clinical conditions. Evidence suggests that higher eating frequency is associated with a lower risk of metabolic syndrome and hypertension (96, 97), while another study indicates that higher eating frequency is associated with blood pressure in adults without cardiovascular disease and diabetes, as well as the progression rate of new-onset hypertension (98). Therefore, larger studies and standardized experimental protocols are needed to determine the association between eating frequency and mortality rates. This could potentially serve as a strategy for daily dietary management in diabetic patients and offer valuable insights into preventing premature mortality from CKM syndrome.

Exercise intervention

In the preliminary management of CKM syndrome, priority should be given to addressing the impact of adverse social determinants of health and improving patients' unhealthy lifestyle habits such as diet and exercise (6). Exercise is a cost-effective lifestyle intervention that can prevent and treat obesity, T2DM, and their complications, and is closely linked to microbiota research (99, 100). A study randomized 39 pre-diabetic patients who had not previously received drug treatment into a sedentary control group and an exercise training group. Significant reductions in weight and obesity were observed across the entire exercise group; Improvements were also noted in insulin sensitivity, lipid profiles, cardiorespiratory health, and levels of adipose factors associated with insulin sensitivity. Further metagenomic sequencing identified significant changes in the relative abundance of *Firmicutes, Bacteroidetes*, and *Proteobacteria*. To understand how exercise-induced changes in gut microbiota regulate host metabolism, KEGG enrichment analysis indicated increased gene abundance in the sedentary group involved in producing phenolic derivatives (indole and p-cresol) and sulfates from aromatic and sulfur-containing amino acids. Subsequently, fecal microbiota from both groups were transplanted into mice, revealing similar trends in body composition, oxygen consumption, and respiratory exchange rates among mice transplanted with feces from exercise participants. Mice receiving fecal transplants from exercise participants showed significant decreases in glucose and insulin levels, marked improvements in glucose handling, and exhibited a microbial metabolite profile distinct from that observed in humans (101). Similarly, another study transplanting feces from exercise participants into mice revealed that metabolites produced by the microbiota could modulate the gut-brain axis to regulate exercise motivation (102).

Strategies for managing gut microbiota

Probiotics

The use of probiotics may help improve the progressive changes in CKM syndrome (103, 104). Probiotics can colonize the human intestinal tract, enhance gut microbiota, regulate metabolism, and maintain intestinal balance. Clinical studies have revealed the positive effects of probiotic interventions on glucose metabolism, particularly the hypoglycemic effects of *lactobacilli* and *bifidobacteria*, which have been confirmed in multiple clinical trials (105, 106). Akbari et al. (107) found that probiotic supplementation significantly reduces insulin resistance, fasting blood glucose, and glycosylated hemoglobin levels. In a clinical study recruiting 38 patients with CKD who used specific probiotic capsules daily for 6 months, the average estimated glomerular filtration rate (eGFR) decline rate significantly slowed from an average monthly decline of 0.54 (-0.18 to -0.91) to 0.00 mL/min/1.73 m^2 (+0.48 to -0.36), and serum levels of inflammatory cytokine and IS were significantly reduced following probiotic use (108). Additionally, a 6-week randomized, placebo-controlled, crossover trial, administering heat-killed *Lactococcus*, *Lactobacillus acidophilus*, and *Bifidobacterium longum* to patients with CKD reduced plasma PCS levels, alleviating cardiovascular damage caused by PBUTs accumulated in CKD. Some preliminary evidence suggests that probiotics may protect cardiovascular metabolism in CKM syndrome (109). For instance, a randomized controlled trial reported beneficial effects of Brewer's yeast in heart failure patients, improving left ventricular ejection fraction (110). However, probiotics administered to critically ill patients with weakened immunity may become opportunistic pathogens causing endocarditis (111), indicating that careful consideration is needed before administering probiotics to vulnerable groups.

Prebiotics

"Prebiotics" refers to substrates selectively utilized by host microorganisms that confer health benefits (112). Prebiotics naturally exist in many fruits, grains, vegetables, honey, and breast milk.

In a single-center, double-blind, placebo-controlled trial involving overweight or obese children aged 7-12 years, participants were randomly assigned to either a prebiotic or placebo group for 16 weeks. Compared to children receiving the placebo, those in the experimental group experienced significant decreases in weight score (3.1% decrease), body fat percentage (2.4% decrease), and trunk fat percentage (3.8% decrease), while the placebo group showed increases (0.5%, 0.05%, and -0.3%, respectively). Additionally, Interleukin 6 levels and triglycerides decreased significantly from baseline in the experimental group, while the placebo group showed a 25% increase. 16S rRNA sequencing indicated a significant increase in *Bifidobacterium* species in the experimental group and a decrease in ordinary pseudo-bacteria species (113). Professor Zhao Liping designed and developed a diet called WTP, consisting primarily of various whole grains, traditional Chinese medicinal food homologues, and prebiotics. Multiple clinical trials have shown that this dietary fiber can significantly enhance the ability of intestinal bacteria in obese and diabetic patients to produce SCFAs. Further studies have demonstrated that similar dietary interventions can improve gut microbiota composition and metabolic health (114).

Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is the most direct method to reshape gut microbiota. Initially recommended for treating recurrent *Clostridioides* difficile infection (115), FMT has also shown efficacy in managing diabetes (116), obesity metabolic syndrome (101), and CKD (117). Anne Vrieze et al. (118) successfully applied FMT to patients with metabolic disorders, finding significantly increased insulin sensitivity in obese patients with metabolic syndrome after gut microbiota infusion from lean donors. In a study by Pieter de Groot's team, 20 diabetic patients were randomly assigned to receive either autologous or allogeneic (healthy donor) FMT. Results showed significantly preserved stimulated C-peptide levels in the autologous FMT group at 12 months. A negative correlation was found between small intestinal *Prevotella* and residual β-cell function (r=-0.55, p=0.02), while plasma metabolites correlated positively with residual β-cell preservation (rho=0.56, p=0.01 and rho=0.46, p=0.042) (119). Early research from our team found significant alleviation of symptoms in diabetic complications following FMT from healthy donor fecal microbiota (120). Animal models, divided into control, CKD, and CKD+FMT groups, showed significant improvement in disrupted gut microbiota, reduced PCS accumulation, and improved glucose tolerance after FMT treatment (121).

FMT therapy is increasingly accepted and recognized as a "natural" therapeutic method for treating various diseases due to its effectiveness, safety, and convenience. It has become a hot topic of interest among biologists, clinicians, and other stakeholders and is rapidly evolving. However, FMT poses unique and complex challenges for clinicians and regulatory agencies, including unclear mechanisms of action, definitions of healthy donors, screening procedures, sample preparation, storage conditions, dosing responses, administration methods, and settings. These factors may limit its broader application in clinical practice and hinder its expansion.

Drug therapy

The AHA has established detailed staging criteria and clinical management recommendations for CKD-MBD syndrome. These guidelines can be utilized for managing CKD-MBD syndrome at different stages, aiming to improve cardiovascular and renal outcomes. For CKD-MBD stages 0 and I, the focus is on lifestyle adjustments to maintain normal weight and other health indicators. For CKD-MBD stage II and beyond, individualized pharmacological treatment is recommended (6).

The AHA recommends considering medications with cardio-renal protective effects to improve gut microbiota and alleviate CKD syndrome outcomes. These medications include angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor blockers (ARBs), glucagon-like peptide-1 receptor agonists (GLP-1RAs), sodium glucose co-transporter 2 inhibitors (SGLT-2is), and novel non-steroidal mineralocorticoid receptor antagonists (MRAs) like finerenone (6, 122–125). A study involving 36 patients treated with ACEIs/ARBs and 19 untreated patients conducted 16S rRNA sequencing and fecal metabolomics analysis. It showed that ACEI/ARB treatment improved gut microbiota by reducing potentially pathogenic bacteria such as *Escherichia coli* and *Klebsiella* spp. and increasing beneficial bacteria like *Odoribacter* spp. Additionally, significant metabolic changes were associated with ACEI/ARB treatment (126). Empagliflozin, an SGLT2i, not only improves hyperglycemia but also reduces weight, lowers blood pressure, and decreases cardiovascular events and mortality (127, 128). Our research team found that the cardiovascular benefits of empagliflozin might be related to changes in gut microbiota and plasma metabolites. In a randomized, open-label, 3-month clinical trial involving 76 newly diagnosed T2DM patients with CVD risk factors, patients were administered either empagliflozin or metformin. Both groups showed significant reductions in HbA1c levels and improvements in glucose metabolism. However, only the empagliflozin group improved cardiovascular disease risk factors, significantly reshaped gut microbiota after one month, elevated levels of SCFAs-producing bacteria such as *Roseburia*, *Ruminococcus*, and *Faecalibacterium* spp., and decreased levels of harmful bacteria including *Escherichia coli-Shigella*, *Bilophila*, and *Hungatella* spp (129). To further understand the primary mechanisms of SGLT2i, non-diabetic and diabetic mice with early and mild hyperglycemia were treated with SGLT2i for one week. This treatment revealed impacts on cardiac and hepatic signaling, with more pronounced effects observed in white adipose tissue, showing increased lipolysis. These effects were particularly influenced by gut microbiota capable of fermenting phenylalanine and tryptophan into cardiovascular PBUTs, with a lower relative abundance of specific bacterial taxa (81).

Similarly, the 2023 ESC Guidelines for managing cardiovascular disease in diabetes patients emphasize the importance of comprehensive management to reduce cardiovascular and renal failure risks, recommending first-line therapies such as non-steroidal MRAs with established cardiovascular and renal benefits (130). Animal studies have shown that compared to spironolactone, finerenone improves myocardial and renal hypertrophy, reduces BNP and proteinuria levels, and decreases the expression of pro-inflammatory and pro-fibrotic genes in cardiac and renal tissues (131).The Phase 3 FIDELIO-DKD and FIGARO-DKD trials confirmed that in patients with T2DM-related CKD, finerenone not only provides renal protection but also improves cardiovascular outcomes. The FIDELITY trial (n = 13,026) further demonstrated the clinical benefits of finerenone, with a 14% reduction in composite cardiovascular risk (HR 0.86, 95% CI: 0.78, 0.95) and a 23% reduction in composite renal outcomes risk (HR 0.77, 95% CI: 0.67, 0.88) (132).

Currently, no studies link gut microbiota and its metabolites to finerenone both domestically and internationally. However, previous research has shown that steroidal mineralocorticoid receptor antagonists can alter the composition and diversity of gut microbiota in hypertensive patients, impacting intestinal barrier permeability and sympathetic nervous system function (133). Therefore, further exploration is needed to determine whether the cardiovascular and renal benefits of finerenone in CKD syndrome patients are mediated through changes in gut microbiota and its toxin-like metabolites as potential targets.

Others

Klotho protein

The Klotho protein is a protective transmembrane protein in the kidneys (134). Animal experiments have shown that IS and PCS can inhibit Klotho expression, activate the renin-angiotensin-aldosterone system (RAAS) and TGF-β pathways, ROS generation, exacerbate oxidative stress and inflammation, and promote renal tubulointerstitial fibrosis formation (135, 136). In a clinical study involving 86 predialysis patients, serum Klotho levels were found to decrease as eGFR declined. Additionally, the interaction between elevated IS levels and increased left ventricular mass was more pronounced in patients with low Klotho levels (137). Serum Klotho levels gradually decrease in CKD patients, while levels of the inflammatory factor Tumor Necrosis Factor-α increase. This significant correlation indicates a close association between reduced Klotho protein and the development of CKD-related microinflammatory states (138).

Studies consistently indicate that Klotho can prevent uremic toxin-related cardiac toxicity by inhibiting oxidative stress to suppress IS-induced endothelial dysfunction (138). Treatment with Klotho protein in a CKD-related left ventricular hypertrophy mouse model significantly inhibited the development of left ventricular hypertrophy (137). Additionally, Klotho protein therapy can prevent IS-induced thrombosis and atherosclerosis in apolipoprotein E knockout mice (139). Therefore, exogenous supplementation of Klotho may be a potential therapeutic approach to inhibit the progression of uremic cardiomyopathy.

Aryl hydrocarbon receptor

Aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor widely expressed in immune, epithelial, endothelial, and stromal cells within barrier tissues. Recent studies indicate that AhR signaling serves as a critical mediator in the progression of diseases induced by various PBUTs, contributing to intestinal homeostasis between the host and gut microbiota (140). All tryptophan metabolites—indole uremic solutes and kynurenic acid—are agonists of the AhR pathway (141, 142). Cardiovascular disease is a leading cause of mortality associated with CKD-MBD syndrome. In a rat model of cardiac hypertrophy, hypertension, and myocardial fibrosis induced by 5/6 nephrectomy, AhR pathway activation was observed, including AhR translocation and downstream protein Cytochrome P450 1(CYP1) expression, accompanied by increased ROS production detected via staining. Experimental evidence demonstrated that IS triggers AhR translocation, leading to significantly increased downstream gene expression, and that AhR inhibitors, CYP1 inhibitors, and AhR-targeting siRNA effectively block ROS production. Moreover, inhibition of the AhR/CYP1/ROS pathway collectively attenuates IS-mediated cardiomyopathy promotion. This research highlights the activation of the AhR/CYP1 pathway in disease, specifically associated with the uremic toxin IS (143). The central role of AhR in the progression of CKD-MBD syndrome and its activation by PBUTs provide compelling evidence supporting AhR as a therapeutic target in the later stages of CKD-MBD. Resveratrol, a natural AhR antagonist, suppresses proteinuria, hypoalbuminemia, and hyperlipidemia in nephritic rats (144). Addi et al. (145) confirmed that CH223191, a specific AhR antagonist, reduces the expression of TF in human endothelial cells during the PBUTs indole-3-acetic acid-mediated process. Meanwhile, Assefa et al. (146) demonstrated that resveratrol attenuates vascular endothelial activation and permeability by blocking the IS/AhR pathway, thereby exerting cardiovascular protective effects.

Despite AhR's potential as a promising clinical target, much of our understanding of its physiological and pathological functions in CKD-MBD syndrome comes from animal models, complicating the translation to clinical applications. Therefore, further research is essential to unravel the complex roles of AhR, ensuring its safe and effective use in prevention and treatment.

AST-120

AST-120 is an oral medication designed to adsorb and remove precursors of PBUTs produced in the gastrointestinal tract. It is currently the only drug known to improve PBUTs symptoms in patients with CKD and delay the need for dialysis (147). AST-120 effectively lowers circulating and renal levels of IS (148, 149). In Japan, it has been widely used in patients with CKD to clear intestinal precursors of PBUTs. Recent case studies by Tomino et al. (150) reported that patients with CKD receiving AST-120 showed an increase in GFR, with kidney function improving rapidly and progressing to ESRD upon discontinuation of treatment. Another study found that AST-120 therapy significantly reduced levels of total IS, PCS, free IS, and free PCS (151). A prospective randomized study on stage II and IV patients with CKD demonstrated that IS levels decreased in the AST-120 treatment group but not in the control group. Besides preserving kidney function and clearing toxins, a retrospective study indicated that AST-120 helped reduce the incidence of cardiovascular events and mortality rates in patients with CKD (152). Additionally, animal experiments have shown that AST-120 can prevent the progression of arteriosclerosis in a mouse model of chronic renal failure by preserving levels of anti-angiogenic factors (153).

Therefore, in addition to traditional drug treatments for CKM and related diseases, therapeutic approaches targeting the gut microbiota primarily include dietary and lifestyle interventions, supplementation with beneficial bacteria, FMT, and research into new drugs. Both supplementation with beneficial bacteria and FMT essentially involve the introduction of exogenous probiotics. Existing studies have shown that they can alleviate disease by increasing the abundance of beneficial gut bacteria, competing for limited nutrients to inhibit the growth of pathogenic bacteria, and reducing the production of PBUTs by pathogenic bacteria. However, its application is limited due to the difficulty of supplementing probiotics in establishing long-term colonization in the host and the risk of transmission of viruses and

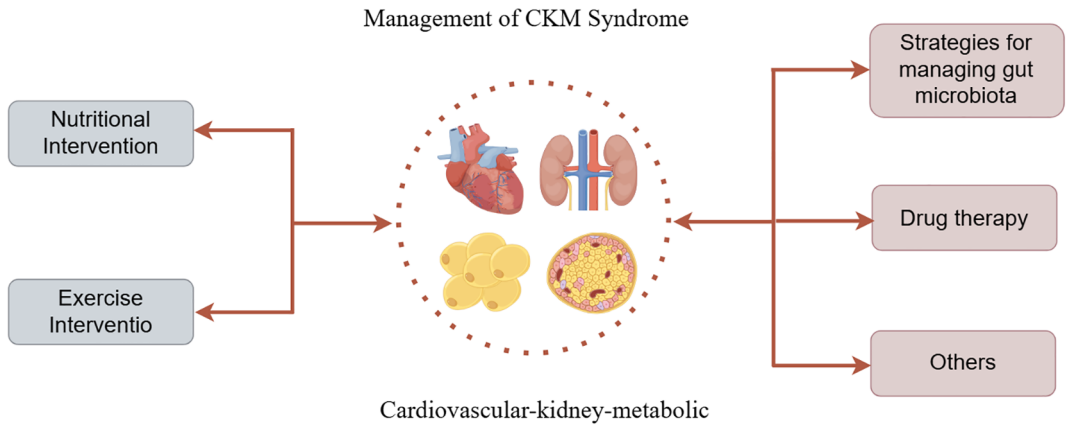

FIGURE 2
Management of CKM syndrome: targeting PBUTs.

diseases from donors. The clinical effect of traditional drug treatment in CKM is not good and the progression of the disease cannot be controlled, so there is a greater need for innovative treatments to reduce PBUTs to maintain the overall state of the disease, but at present, a large number of clinical experimental studies and validations are needed. In conclusion, the treatment of PBUTs needs to be developed urgently (Figure 2).

Prospect

The AHA report emphasizes that CKM syndrome is a systemic and progressive pathophysiological process. It is not merely an aggregation of several diseases, but rather a result of the mutual influences and promotion among metabolic diseases such as diabetes, CKD, and CVD. As the disease progresses, the accumulation of PBUTs generated through metabolism exacerbates these conditions, ultimately leading to cardiorenal damage. Therefore, early detection and prevention are crucial in treating CKM syndrome. Clinical practitioners need a clear understanding of CKM syndrome and should actively screen at-risk populations. Developing new screening and risk prediction models for CKM syndrome will help prevent CVD events associated with it, significantly delaying its progression and improving patient survival rates. Our preliminary cross-sectional study found distinct gut microbiota characteristics in healthy individuals, early-stage patients with DKD, and late-stage patients with DKD, with variations in core genome content associated with toxin production. We identified 54 core genomes capable of significantly distinguishing patients with DKD from healthy individuals and demonstrating good discriminatory ability among patients with DKD of different severities (32). Therefore, gut microbiota and PBUTs hold promise as a CKM syndrome screening and risk prediction model, addressing early screening challenges and enabling proactive prevention and treatment to avoid adverse cardiorenal outcomes.

PBUTs, characteristic of the CKM syndrome environment, have been the focus of numerous experimental and clinical approaches aimed at mitigating their pathogenic effects and halting the progression of cardiorenal outcomes. However, further discussion is needed to evaluate whether these methods can reverse the CKM syndrome state and their impact on prognosis. Additionally, detailed staging of CKM syndrome requires the adoption of multiple modalities to adjust clinical management across different stages, facilitating precise, targeted therapy against these toxins.

The introduction of the novel concept of CKM syndrome represents not only a redefinition of the disease status but also a comprehensive update of treatment paradigms. Addressing PBUTs in the treatment of CKM syndrome offers a promising approach to overcoming challenges in screening, predicting, and managing the progression of CKM syndrome. This approach aims for the integrated, holistic management of patients with CKM syndrome.

Author contributions

SZ: Writing – original draft, Writing – review & editing. ST: Writing – original draft, Writing – review & editing. YL: Writing – original draft. BX: Writing – original draft. QX: Writing – review & editing. LZ: Writing – review & editing. HY: Writing – original draft, 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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Gut microbiota-derived imidazole propionate: an emerging target for the prevention and treatment of cardiometabolic diseases

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Despite significant advancements in prevention and treatment, cardiometabolic diseases continue to pose a high burden of incidence and mortality. The chronic progression of these diseases necessitates the identification of early and complementary therapeutic targets to elucidate and mitigate residual risks in patient care. The gut microbiota acts as a sentinel between internal and external environments, transmitting modified risks associated with these factors to the host. Imidazole propionate (ImP), a histidine metabolite originating from the gut microbiota, gained attention after being found to impair glucose tolerance and insulin signaling several years ago. Epidemiological studies over the past five years have demonstrated a robust correlation between ImP and an increased risk of onset of type 2 diabetes (T2D) and obesity, exacerbation of kidney traits in chronic kidney disease (CKD), progression of atherosclerotic plaques, and elevated mortality rates in heart failure (HF). These findings suggest that ImP may serve as a pivotal target for the prevention and treatment of cardiometabolic diseases. Mechanistic insights have uncovered associations between ImP and insulin resistance, impaired glucose metabolism, chronic inflammation, and intestinal barrier damage. This review provides a comprehensive summary of the current evidence regarding the association between ImP and cardiometabolic impairment, highlighting its potential in advancing personalized approaches to disease prevention and management, and

exploring the intricate interplay of diet, gut microbiota, and ImP in cardiovascular metabolic impairment. Overall, this review offers valuable insights into the multifaceted roles of ImP in cardiometabolic diseases, identifies current knowledge gaps, and discusses future research directions.

KEYWORDS

imidazole propionate, cardiometabolic disease, diabetes, microbiome, microbial metabolites, biomarker

1 Introduction

Cardiometabolic diseases impose a significant global health burden, surpassing other disorders in terms of morbidity and mortality, with projections indicating a sharp increase over the next 25 years (1, 2). This category encompasses a range of chronic conditions affecting both cardiovascular and metabolic health, including cardiovascular disease (CVD), insulin resistance, obesity, diabetes, chronic kidney disease (CKD), and nonalcoholic fatty liver disease (NAFLD) (3, 4). Managing these diseases presents challenges for healthcare providers due to their often-asymptomatic nature until advanced stages, highlighting the pressing need for more effective prevention and intervention strategies.

Accumulating evidence implicates imbalances or compositional changes in intestinal microbes in both physiological and pathological alterations in the host (5). The causal contribution of gut microbiota to cardiometabolic diseases is further supported by a plethora of direct experimental evidence (6). A pivotal mechanism involves the production of small molecules by gut microbes, capable of exerting effects at or beyond the host gut barrier. Initially, research primarily focused on bile acids, short-chain fatty acids (SCFAs), branched-chain amino acids, and carnitine-derived metabolites (7–10). Advances in metabolomics have facilitated the identification of increasingly crucial intestinal metabolites, hastening the discovery of potential biomarkers to enhance the diagnosis and prognosis estimation of various diseases.

Recently, imidazole propionate (ImP), a histidine-derived metabolite produced by gut microbes, has garnered increasing attention for its close correlation with metabolic disorder. The investigation into ImP's role in human disease traces back to 1972 when it was discovered to be excreted by patients with intestinal disorders. Interestingly, it was almost absent in feces and urine from healthy subjects, suggesting ImP's potential as a microbial metabolite with adverse health effects (11). However, for a considerable period thereafter, ImP seemed to fade into obscurity. It wasn't until 2018 when researchers from the University of Gothenburg and Sahlgrenska University Hospital (12) discovered its association with impaired insulin signaling in mice and humans that ImP came back into the spotlight. Subsequently, increasing clinical studies reveled close links between circulating ImP levels and metabolic disorder and CVD, including type 2 diabetes (T2D) (13), blood pressure (14), obesity (15), non–alcoholic steatohepatitis (NASH) (16), CKD (17), artery atherosclerosis (18–20), and heart failure (HF) (21). Supplementation with ImP has been demonstrated to exacerbate glucose intolerance (12), impair wound healing (22), and compromise the integrity of the intestinal barrier (23) in mice.

Here, we comprehensively review the available evidence on the biological effects of ImP, emphasizing its potential therapeutic applications as a target for treating cardiometabolic diseases, and discuss future research directions.

2 Synthesis and metabolism of ImP in mammals

ImP, also referred to as dihydrourocanate or deamino-histidine, arises from the metabolic activity of gut microbiota on dietary histidine. Histidine, an essential amino acid obtained from the host diet, serves as a fundamental substrate for protein synthesis and acts as a precursor for the biogenic amine histamine, catalyzed by histidine decarboxylase. Moreover, surplus histidine undergoes metabolic conversion to trans-urocanate via histidine ammonia-lyase (EC:4.3.1.3, encoded by the hutH gene) (24). Subsequently, trans-urocanate is primarily metabolized to cis-urocanate in the skin and to glutamate and NH3 in the liver (24, 25). In the colon, urocanate reductase (EC:1.3.99.33, encoded by the urdA gene), produced by the intestinal microbiota, facilitates the reduction of trans-urocanate into the non-metabolizable product, ImP (26, 27). Ultimately, ImP is excreted either directly through feces or absorbed by the intestines and subsequently excreted through urine (11).

Under physiological conditions, circulating ImP levels exhibit minimal individual variation, ranging from a few to several tens of

Abbreviations: AMPK, adenosine 5′-monophosphate-activated protein kinase; BMI, body mass index; CAD, coronary artery disease; CDAHFD, choline-deficient amino acid-defined high-fat diet; CKD, chronic kidney disease; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HF, heart failure; ImP, imidazole propionate; IRS1, insulin receptor substrates 1; IRS2, insulin receptor substrates 2; MAPK, mitogen-activated protein kinase; MetaCardis, European multicentric cohort; mTORC1, mechanistic target of rapamycin complex 1; NAFLD, non-alcoholic fatty liver disease; NASH, non –alcoholic steatohepatitis; r, Pearson correlation coefficient; r_s, Spearman rank correlation coefficient; SCFAs, short-chain fatty acids; T2D, type 2 diabetes; TLR4, toll-like receptor 4; UAC, urinary albumin-to-creatinine.

nanomolars (12–14). However, under pathological conditions such as T2D and CVD, its concentration can escalate by approximately a hundredfold (12, 13).

Despite deriving from histidine metabolism, ImP levels in the body are not determined by histidine intake (13), but are primarily affected by enzyme activity and the composition of intestinal microbiota (12, 13, 26). Large-scale screening for UrdA, which encodes the urocanate reductase responsible for ImP production, has identified bacteria harboring "Y" or "M" UrdA homologs as authentic ImP producers from urocanate (12). Urocanate reductase exhibits optimal activity at neutral pH (12, 26, 28). These UrdA-containing bacteria- encompass species such as *Aerococcus urinae*, *Streptococcus mutans*, *Anaerococcus prevotii*, *Adlercreutzia quolifaciens*, *Eggerthella lenta*, *Lactobacillus paraplantarum*, *Brevibacillus laterosporus*, and *Shewanella oneidensis* (12). Over the past five years, an increasing number of intestinal bacteria have been implicated in direct or indirect associations with ImP production, as summarized in Table 1.

3 Role of ImP on cardiometabolic diseases

3.1 ImP: linking insulin resistance, type 2 diabetes risk, and metformin inhibition

Bacterial metabolites originating in the gut traverse to the liver via the portal vein before entering systemic circulation. The crosstalk between the gut and liver ultimately leaded to insulin resistance and even diabetes (29). Koh et al. investigated amino acid-derived microbial metabolites potentially linked to insulin resistance and T2D (12). In their initial study involving 15 obese subjects (body mass index [BMI] > 40), higher concentrations of ImP were observed in both portal and peripheral blood of 5 T2D subjects compared to 10 BMI-matched controls. This finding was corroborated in a larger cohort of 649 middle-aged individuals from the Swedish community, where ImP levels remained significantly elevated in treatment-naive T2D subjects after adjusting for BMI, sex, and age (12). Subsequently, numerous studies successively reported the association between ImP levels and T2D. In a large European multicentric cohort (MetaCardis) comprising 1,958 subjects from France, Germany, and Denmark, progressively elevated ImP levels were observed across patients with normal glucose tolerance, prediabetes, and overt T2D (13). Additionally, circulating ImP levels exhibited positive correlations with HbA1C (13, 30), HOMA-IR (13), insulinemia (13), fasting glucose (13, 15) and postprandial glucose (13, 31). These findings indicate that ImP is not only associated with impaired glucose metabolism but also with diabetic status.

The analysis of gut microbiota in T2D patients effectively addressed the reasons for the changes in circulating ImP levels. In an *in vitro* gut simulator experiment monitoring ImP production kinetics, the gut microbiota of T2D patients demonstrated ImP production capability, unlike non-T2D patients (12). Further investigation revealed the enrichment of ImP-producing bacteria, characterized by "Y" or "M" UrdA homologs, in the intestines of T2D patients (12), as well as in the intestine and skin of T2D mice (22). These included strains previously associated with an elevated

TABLE 1 Association of microbial features with imidazole propionate production.

Association with ImP	Microbial Features (Indicators or Bacteria)	Reference
ImP-producing bacteria	The bacteria with "Y"- or "M"-UrdA homologs: *Aerococcus urinae, Streptococcus mutans, Anaerococcus prevotii, Adlercreutzia quolifaciens, Eggerthella lenta, Lactobacillus paraplantarum, Brevibacillus laterosporus, Shewanella oneidensis.*	(12)
Positively correlated with ImP levels	Bacteroides 2 enterotype	(13)
	Lactobacillus: *L. gasseri, L. fermentum, L. plantarum, L. amylovorus, L. crispatus, L. iners.*	(12, 13, 17, 18, 20, 30, 71, 87)
	Streptococcus: *S. mutans, S. parasanguinis, S. gallolyticus, S. anginosus, S. oralis, S. gordonii, S. agalactiae.*	
	Clostridium: *C. bolteae, C. symbiosum, C. ramosum, C. scindens, C. bartlettii, C. spiroforme, C. clostridioforme, Flavonifractor plautii.*	
	Bifidobacterium: *B. longum, B. dentium, B. breve, B. reuteri*	
	Bacteroides: *B. xylanisolvens, B. vatus, B. dorei vulgatus, B. faecis.*	
	Others: *Eggerthella lenta, Veillonella parvula, Veillonella atypica, Dialister invisus, Ruminococcus gnavus, Pseudoflavonifractor capillosus, Gardnerella vaginalis, Peptostreptococcus stomatis, Fusobacterium nucleatum, Parabacteroides gordonii, Blautia hansenii, Citrobacter freundii, Pediococcus acidilactici, Escherichia-Shigella, Allisonella, Collinsella, Desulfovibrio*	
Negatively correlated with ImP levels	Microbiome gene count (607,000 threshold)	(13)
	Roseburia: *Roseburia intestinalis, Roseburia hominis,*	(12, 13, 17, 18, 20, 30, 71, 87)
	Clostridium: *C. sp. CAG:91, C. sp. CAG:122, C. sp. CAG:127, C. sp. CAG:264.*	
	Others: *Clostridioides difficile, Coprococcus comes, Eubacterium eligens, Faecalibacterium prausnitzii, Dorea formicigenerans, Subdoligranulum variabile, Fournierella massiliensis, Phascolarctobacterium, Anaerostipes, Faecalibacterium, Subdoligranulum variabile.*	

risk of T2D in large population cohorts, such as *Streptococcus mutans* (32), *Eggerthella lenta* (33), and *Lactobacillus gasseri* (33).

However, ImP serves not only as a disease marker but also correlates with an increased risk of prediabetes and T2D, as demonstrated in cohort studies (13, 15), suggesting its biological impact on T2D progression. In animal experiments, ImP injection induced glucose intolerance and decreased hepatic insulin signaling (12). Mechanistically, ImP disrupts insulin signaling by activating p38γ mitogen-activated protein kinase (MAPK), leading to p62 phosphorylation and subsequent activation of mechanistic target of rapamycin complex 1 (mTORC1). This results in the phosphorylation and degradation of insulin receptor substrates 1 and 2 (IRS1 and IRS2). Consistently, phosphorylation of p62 and S6K1 were elevated in the human liver compared to healthy controls (12), highlighting the role of ImP in impairing insulin signaling through the p62/mTORC1 pathway.

In addition to its impact on T2D itself, ImP has also been found to influence the hypoglycemic effects of metformin, the first-line therapy for T2D. Metformin exhibits substantial variability in efficacy among individuals, and genetic variations, particularly in genes encoding transporters such as organic cation transporter 1 (OCT1) (34) and glucose transporter 2 (GLUT2) (35), have been identified as influencing metformin response. In addition to gene polymorphisms, Koh et al. discovered that intestinal ImP levels contribute to this variability (36). T2D patients on metformin with persistently high blood glucose levels showed elevated ImP concentrations.

Further experimental studies demonstrated that ImP diminishes the glucose-lowering effect of metformin and inhibits metformin-induced activation of adenosine 5′-monophosphate-activated protein kinase (AMPK) by impeding AMPK serine phosphorylation through the p38γ/Akt pathway. However, as the authors point out, the study has certain limitations worth noting. Given its cross-sectional design, it remains unclear whether individuals with higher blood glucose values (and consequently higher plasma ImP levels) actually responded poorly to metformin or had more severe diabetes prior to treatment initiation. Hence, a longitudinal cohort study is warranted to ascertain whether ImP directly undermines metformin efficacy and whether metformin contributes to the proliferation of ImP-producing bacteria.

3.2 ImP: implicated in atherosclerosis through chronic systemic inflammation and immune activation

The sub-analysis of the MetaCardis study, including 20% of participants with CVD, revealed a significant increase in circulating ImP concentrations among CVD patients after adjusting for traditional risk factors (age, gender, BMI, ethnicity), kidney function, and presence of T2D (13). Correlation analysis demonstrated a strong positive correlation between ImP levels and serum inflammatory markers, including total leukocyte count, high-sensitivity C-reactive protein (hs-CRP), and interferon gamma-induced protein 10 (IP-10) (13), suggesting a potential association between ImP, inflammation, and CVD progression, warranting further investigation.

Recent large-scale clinical studies have linked ImP to atherosclerosis (18–20), a chronic inflammatory vascular disease and the major cause of CVD (37). The role of gut microbiota in atherosclerosis has been supported by increasing mechanistic evidence (38). In fact, significant changes in gut microbiota have been observed in patients with subclinical coronary atherosclerosis before plaque formation. A study involving 8, 973 participants without overt atherosclerotic disease revealed significant alterations in gut microbiota, including oral microbial species like *Streptococcus* spp, which correlated significantly with ImP levels and systemic inflammation markers (hs-CRP levels and neutrophil counts) (20). However, due to its cross-sectional design and lack of experimental evidence, the study failed to extrapolate the predictive value of ImP on plaque formation and its potential implications in atherogenesis.

Nevertheless, data from HIV patients have provided indications of a possible association between ImP and the presence of plaques in carotid and coronary arteries (18, 19). HIV infection is linked to chronic inflammation and immune activation, critical factors in atherosclerosis and thrombosis development (39). HIV-induced disruptions in gut microbiota exacerbate chronic inflammation and metabolic irregularities, increasing atherosclerosis risk (40, 41).

In a study of 320 females living with or at risk of HIV infection, with 26% having carotid artery plaque, a distinct shift in gut microbiota composition and increased ImP plasma levels were observed (18). Circulating ImP levels were inversely correlated with potentially beneficial microbial species linked to reduced carotid artery plaque. Additionally, ImP levels positively correlated with serum inflammatory markers, including CX3CL1, TNFSRF9, and LIF-R, associated with immune activation and inflammation pathways related to atherosclerotic plaques (42–44). Notably, after adjusting for plasma ImP levels or inflammatory markers, the association between gut bacterial species and plaque weakened, indicating that circulating ImP levels and related inflammatory markers may partly explain these associations. Further analysis identified 17 ImP-associated species (as illustrated in Table 1), with 8 correlating with the functional enzyme hutH. A gut microbiota score derived from these ImP-associated species showed a positive correlation with plaque formation and several pro-inflammatory markers, even after adjusting for multiple factors. Collectively, gut bacteria may contribute to plaque formation by modulating host immune activation and inflammation through elevated IMP levels.

Recent research suggests that ImP is not only associated with plaque formation but also with plaque obstruction. HIV-infected patients with obstructive coronary artery disease (CAD) exhibited lower gut microbiota diversity and significant compositional changes compared to HIV-infected individuals without CAD or non-obstructive CAD, with increased abundance of known ImP producers such as *Rumiococcus gnavus* and *Veillonella* (19). ImP plasma levels were associated with this dysbiosis, significantly elevated in participants with obstructive CAD (19). However, after adjustment for traditional and HIV-related risk factors, gut

dysbiosis but not plasma ImP was independently associated with obstructive CAD, indicating that the effects of gut microbiota may extend beyond ImP in plaque obstruction. Longitudinal studies are needed to establish a causal relationship between ImP levels and plaque formation, shedding light on its predictive value for atherosclerosis. Additionally, exploring ImP in the context of HIV-related CVD is crucial, highlighting the importance of understanding the role of ImP and gut dysbiosis in driving CVD risk among HIV patients.

The recent findings offer a strong foundation for future investigations, yet additional experiments are necessary to move beyond associations and establish causal evidence linking ImP to atherosclerosis. Firstly, further research is needed to elucidate the mechanistic connection between ImP and inflammation, addressing the significant correlations observed in multiple studies. Secondly, Atherosclerosis initiates with endothelial injury, leading to the accumulation of macrophage foam cells and infiltration of smooth muscle cells, resulting in fatty streak formation (45). Inflammatory processes play a pivotal role in the development of vulnerable plaques (46). Rupture of the plaque's cap triggers platelet aggregation, precipitating thrombosis and vascular obstruction (47). Therefore, understanding whether ImP is involved in endothelial injury, foam cell formation, and platelet aggregation should be the central focus of future research into the progression of atherosclerosis.

3.3 ImP independently predicts incident heart failure and mortality

Individuals with T2D face more than a twofold increased risk of developing HF compared to non-T2D patients, with higher risks of incident cases and mortality among diagnosed patients (48). Profiling the metabolic connections and shared components of T2D and HF could unveil new disease pathways, enhance risk prediction, and enable tailored prevention and management strategies (49). The elevation of intestinal metabolite ImP in the circulation of T2D patients, along with its induction of insulin resistance in animal models (12), offers a novel perspective on unraveling the molecular signatures and metabolic remodeling of HF and its associated factors.

Targeted metabolomic analysis of 260 individuals with diverse glucose metabolism from the Risk Evaluation and Management of Heart Failure (REM-HF) cohort in China identified ImP as a microbial signature contributing to the shared etiologies of T2D, HF, and CKD (21). Data from the Boston Puerto Rican Health Study (BPRHS) cohort (50) and European Prospective Investigation into Cancer (EPIC)-Norfolk study (49) further corroborated this finding. Impressively, serum ImP levels increased by 1.1–1.6 fold with each additional chronic HF comorbidity (21), supporting ImP as a component of the metabolic connections among T2D, HF, and CKD.

Recently, Molinaro et al. from Sweden (51) investigated the association between circulating ImP levels, HF, and incident mortality risk. In the population-based MetaCardis cohort, significantly higher ImP levels were observed in individuals with established CVD or HF compared to those without, with the highest levels detected in HF patients. Individuals in the highest quartile of ImP levels had a threefold increased risk of HF compared to those in the lowest quartile, even after adjusting for multiple traditional cardiovascular risk factors. Moreover, ImP levels were inversely associated with left ventricular ejection fraction (LVEF) and positively correlated with pro-atrial natriuretic peptide (proANP) and N-terminal pro–B-type natriuretic peptide (NT-proBNP) levels. These findings were consistent across the GeneBank cohort from North America, predominantly comprising patients with HF (n = 407), CVD (n = 1,331), and without CVD or HF (n = 417). Additionally, longitudinal follow-up data from the North American cohort revealed that the highest quartile of ImP was independently associated with an increased risk of overall mortality, even after adjusting for traditional risk factors and baseline covariates (adjusted HR = 1.85, 95% CI [1.20, 2.88], $P < 0.01$). Overall, this study, drawing from two large independent cohorts, offers compelling evidence supporting a substantial correlation between ImP levels and CVD, HF, and HF-associated phenotypes including reduced left ventricular ejection fraction and heightened natriuretic peptide levels. Crucially, this correlation persists regardless of obesity and T2D, known contributors to disease progression. *In vitro* experiments conducted on H9c2 cardiomyoblast cells pretreated with hypoxia/reoxygenation further supported a causal link between ImP and distinct HF-relevant phenotypes. In this study, the intervention of 0.1 μM ImP for 24 hours significantly elevated the expression of the Natriuretic Peptide B gene (NPPB), which encodes the B-type natriuretic peptide (BNP), and disrupted cardiomyoblast functions, as indicated by significantly reduced mitochondrial membrane potential (21).

The current research on the association between elevated circulating ImP levels and HF presents valuable insights, yet it also reveals several limitations and areas for future investigation. One major concern is the lack of clarity regarding the underlying reasons for the elevation of ImP in HF patients, posing a significant gap in our understanding. Moreover, the reliance on cross-sectional data underscores the need for longitudinal research to establish definitive causal relationships. Furthermore, the predominantly focused research on specific populations calls for more diverse cohorts to ensure the generalizability of findings. Future studies should prioritize exploring the mechanistic understanding of ImP's role in HF development and conducting interventional trials to assess the therapeutic potential of targeting ImP levels. Consideration of confounding factors such as medication use and lifestyle variables is essential, and efforts to validate ImP as a diagnostic and prognostic biomarker are warranted. Addressing these gaps through comprehensive research endeavors will enhance our understanding of the involvement of ImP in HF and facilitate the development of effective therapeutic strategies.

3.4 ImP: a novel factor associated with nonalcoholic fatty liver disease

NAFLD, affecting approximately one quarter of the global population, encompasses a spectrum of conditions ranging from

simple hepatic steatosis, often linked to obesity, to NASH, which can progress to fibrosis, cirrhosis, and hepatocellular carcinoma (52). The gut and liver are interconnected through the portal vein, forming the gut-liver axis, which serves as a direct pathway for gut microbiota and their metabolic by-products to reach the liver (53). Bidirectional communication along the gut-liver axis plays a pivotal role in NAFLD pathogenesis (54). In NAFLD, microbial dysbiosis in the gut, particularly a decrease in SCFAs-producing microbiota, has been documented (55). This reduction in SCFAs production leads to an elevation in intestinal pH (56), influencing bacterial metabolite production and subsequent absorption into the host circulation (57).

In Göttingen minipigs fed a choline-deficient amino acid-defined high-fat diet (CDAHFD), serving as a NASH animal model, notable increases in serum ImP concentration and pancreatic glucagon levels were observed. These changes were accompanied by liver activation of mTORC1, as indicated by increased expression of liver RHEB and MTOR genes, along with impaired hepatic insulin signaling, demonstrated by decreased expression of IRS1 and IRS2 (16). Moreover, multiple linear regression analysis identified ImP as a statistically significant predictor for glucagon levels (P = 0.0068). Additionally, 16S rRNA analysis showed significant downregulation of intestinal SCFAs-producing bacteria in CDAHFD-fed minipigs, including butyrate-producing members of *Lachnospiraceae* and propionate producers from the *Muribaculaceae* family (16). This resulted in an elevation of colon luminal pH, creating an environment conducive to enzymatic activity of bacterial urocanate reductase, thereby facilitating ImP production from histidine metabolism (12, 16, 26, 28). Following production, ImP is likely transported from the intestines to the liver via the portal vein (58), where it subsequently contributes to impaired hepatic insulin signaling, hyperglucagonemia, decreased expression of the glucagon receptor, and disruption of the liver-α-cell axis (16). This aligns with emerging evidence showing a negative correlation between ImP levels and fibroblast growth factor 21 (FGF-21), an endogenous regulator of lipid and glucose metabolism (30). Recently, ImP were enriched in cirrhotic patients with chronic hepatitis B compared to healthy subjects, signifying its potential role in chronic hepatitis B progression, but its role remains to be further clarified.

Future research should delve into elucidating the exact role of ImP in the pathogenesis of NAFLD and other liver disease. Specifically, efforts should focus on determining the association of ImP with the progression of liver diseases and its potential diagnostic and therapeutic implications. Moreover, researchers could explore strategies to modulate ImP levels by manipulating gut microbiota composition or intervening in gut SCFAs production, thereby developing novel treatment approaches.

3.5 ImP: a promising target for chronic kidney disease

Globally, over 10% of the population suffers from CKD, characterized by kidney damage, typically indicated by urinary albumin, or decreased kidney function, measured by glomerular filtration rate (59). Individuals with CKD face a significantly elevated risk of CVD and cardiovascular-related mortality (60), underscoring the importance of early detection for effective management. The gut microbiota and their associated metabolites play a crucial role in the microbiota-gut-kidney axis, offering a promising avenue for early diagnosis and personalized treatment to slow renal progression (61).

Recent studies have identified ImP as another metabolite linked to kidney traits, prospectively associated with CKD incidence over time (17). In a large study involving 2,438 Hispanic/Latino adults (12% with CKD), elevated ImP levels were correlated with worsening kidney traits, including reduced eGFR, increased urinary albumin-to-creatinine (UAC) ratio, and CKD incidence over approximately 6 years (17). Similarly, in another cohort from China with varying glucose tolerances, ImP levels exhibited a strong association with creatinine, Cystatin C, and estimated glomerular filtration rate (eGFR). Furthermore, serum ImP levels increased by 1.5 times with the occurrence of CKD in patients with T2D and chronic HF (21). Notably, ImP was more strongly associated with biomarkers of CKD than with those of HF and T2D, indicating its potential pathogenic role in all three conditions and suggesting shared etiologies mediated by ImP among these diseases.

The understanding of the mechanisms underlying increased ImP levels in CKD is still evolving. Alterations in the gut microbiota may be a contributing factor, as CKD progression leads to factors such as sodium and water retention, increased circulatory system pressure, visceral congestion, intestinal wall edema, and impaired intestinal barrier function, resulting in bacterial translocation and gut dysbiosis (62). Studies like the Hispanic Community Health Study/Study of Latinos (HCHS/SOL) study (17) have shown that higher CKD incidence rates and UAC ratios, along with lower eGFR, are associated with reduced gut microbiota diversity and alterations in overall microbial composition, which may directly contribute to increased ImP production. Future research should further explore the relationship between CKD progression, gut dysbiosis, and ImP production to uncover potential therapeutic strategies for managing CKD-related complications and reducing the risk of ImP-associated health issues.

Another interesting discovery is the observed prospective association of ImP with changes in renal function and impairment in CKD, particularly prominent in patients with diabetes (17). This is consistent with animal studies where serum ImP levels were significantly elevated in diabetic mice models (63). ImP levels positively correlated with renal functional parameters like the UAC ratio. In cellular studies, ImP was found to stimulate inflammation and fibrosis by promoting toll-like receptor 4 (TLR4)-mediated phosphorylation of NF-κB and Stat3 and expression of IL-6, TGF-β1, and MyD88 (63). Moreover, downregulating ImP-producing bacteria, such as certain genera of *Bacteroides* and *Unidentified Ruminococcaceae*, was shown to ameliorate diabetic kidney disease by suppressing ImP-induced protein expression of the TLR4 signaling pathway *in vivo* and *in vitro* (63). Understanding the mechanistic role of ImP in CKD progression and its associations with other renal conditions, along with validating these mechanisms

through extensive cell and animal model studies, could lead to improved clinical applications in the future.

3.6 ImP: a potential biomarker for obesity and its association with blood pressure

The interconnection among obesity, hypertension, diabetes, and CVD underscores the importance of investigating shared metabolic pathways to improve risk assessment and develop personalized prevention and management strategies. Studies exploring the association between ImP, T2D, and CVD, such as those by Koh and Molinaro, et al. (12, 13) have included patients with overt metabolic diseases or CVD, complicating the differentiation of ImP's correlation with known risk factors like weight gain, hypertension, and cholesterol. Insights from microbiome community typing analyses within the MetaCardis cohort provided some clues, revealing elevated ImP levels in individuals with the Bacteroides2 (Bact2) enterotype (13), a gut microbiota profile associated with systemic inflammation and obesity (64). Research involving 1,018 females from the UK Adult Twin Registry (TwinsUK) cohort further supports the correlation between ImP and obesity (15). This study found that serum ImP levels were positively correlated with BMI (Pearson correlation coefficient [r] = 0.18, P = 5E-9), visceral fat mass (r = 0.067, P = 0.06), and an increased risk of obesity (r = 0.2, P = 8E-9), indicating circulating ImP levels as a potential marker for obesity.

In another cohort of overweight/obese subjects without T2D and not on any CVD medication, the association between circulating plasma ImP concentrations and CVD risk factors, including blood pressure, HDL cholesterol, and LDL cholesterol, was investigated (14). This study revealed a positive correlation between plasma ImP concentrations and diastolic blood pressure (Spearman rank correlation coefficient [rs] = 0.285, P = 0.004), with borderline significance for systolic blood pressure (rs = 0.187, P = 0.060). Interestingly, no significant association was found between plasma ImP concentrations and peripheral or hepatic insulin resistance, contrary to the findings of Koh et al. (12). This discrepancy could be attributed to differences in the study populations, as Koh et al.'s study included patients with varying BMI and metabolic disease severity. However, the data revealing the association between ImP and blood pressure comes from cohort comprising subjects without T2D, with a very homogeneous range of BMI, and without any medication or overt chronic diseases except for metabolic syndrome (14), suggests that if ImP influences blood pressure, it may do so through mechanisms other than insulin resistance.

While the association between ImP and obesity, as well as blood pressure has been observed, the underlying mechanisms remain unclear. Future research should delve deeper into the molecular pathways linking ImP to obesity and hypertension and explore potential therapeutic targets to mitigate its effects on cardiovascular health. Additionally, the discrepancy in findings regarding the association between ImP and insulin resistance highlights the need for further clarification through well-controlled studies involving diverse populations.

4 Reduced ImP for cardiometabolic benefits? - insights from diet and gut health

Unhealthy dietary patterns, such as the contemporary Western diet characterized by low fiber content and high levels of animal proteins, saturated fats, sodium, and sugar, have been strongly linked to cardiometabolic disorders (65, 66). Conversely, diets rich in fiber and vegetable proteins, such as the Mediterranean, vegetarian, or plant-based low-protein diets, have shown metabolic benefits (67, 68). Increasing evidence suggests that metabolites produced by gut microbiota play a crucial role in mediating the effects of dietary patterns on host metabolism (69). In the MetaCardis study, circulating ImP levels were positively correlated with saturated fat intake (primarily driven by high cheese consumption) and negatively correlated with fiber and unsaturated fat intake (due to increased consumption of vegetables and nuts) (13). ImP levels were also inversely associated with dietary quality indices like the Alternate Healthy Eating Index, dietary diversity score, and Mediterranean diet scores (13). In a two-week dietary intervention study involving healthy individuals, transitioning from a Western diet to one rich in fiber, fruits, vegetables, and protein led to increased creatinine-normalized urinary ImP levels (70). Similarly, another intervention study focusing on subjects with HbA1c levels exceeding 6% showed that intake of resistant maltodextrin (a type of dietary fiber) reduced fecal ImP levels, particularly in individuals with elevated ImP levels before intervention (71). Overall, dietary fiber intake, unsaturated fat consumption, and adherence to healthy dietary patterns may inversely correlate with glucose metabolism disorders by reducing ImP levels.

Healthy dietary patterns, including high-fiber diets, are associated with greater intestinal microbial diversity and bacterial gene richness (72–74). Decreased intestinal microbial diversity and bacterial gene richness have been linked to metabolic disturbances (75–77). ImP has emerged as a circulating metabolite reflective of gut microbiome α diversity metrics, with a Shannon diversity index of approximately -0.2 ($P < 0.01$) (15), and is elevated in individuals with low bacterial gene richness (13). Thus, ImP may serve as an important biomarker reflecting the combined influence of diet, gut microbiota, and genetic diversity on cardiometabolic health.

As a natural source of SCFAs, slight differences in dietary fiber structure can lead to distinct effects on gut microbiome composition, resulting in targeted shifts in the production of SCFAs (78). Increased consumption of vegetables and fruits has been associated with higher abundance of SCFA-producing bacteria. For instance, fruit and vegetable intakes were positively associated with *Coprococcus* species, *Faecalibacterium prausnitzii*, *Roseburia hominis*, and *Firmicutes bacterium CAG:95* across multiple studies (79–83), which have been reported to have a significant negative correlation with ImP production, as depicted in Table 1. Additionally, studies indicate that decreased SCFA production leads to an increase in intestinal pH (16, 56), providing an optimal environment for urocanate reductase, the bacteria responsible for ImP production, to exert maximal activity. While current evidence is limited, given the observed link between

decreased SCFAs and metabolic impairments in numerous studies, this hypothesis seems plausible, suggesting that ImP could serve as a biomarker of dysregulated gut microbiome—due to an unhealthy diet or disease. Therefore, implementing dietary modifications to promote healthier eating habits, along with interventions targeting the regulation of intestinal microbiota composition—such as fecal microbiota transplantation, specific microbiota transplantation or supplementation with probiotics/prebiotics—may offer effective strategies to reduce ImP levels. However, significant gaps remain in understanding the mechanistic relationship between ImP, SCFA production, gut microbiota composition, and intestinal environment. Future research endeavors should aim to elucidate these mechanisms and explore clinical interventions targeting dietary adjustments and microbiota modulation to mitigate ImP-related cardiometabolic risks.

5 Knowledge gaps and future perspectives

5.1 Development of novel drug targets associated with ImP

The association between elevated ImP levels and cardiovascular metabolic impairment underscores the importance of exploring the mechanisms underlying ImP's effects, which may elucidate the potential for targeting ImP to improve cardiovascular metabolism and identify novel drug targets. Insights from the work of Koh and Molinaro et al. (12, 13, 36) have shed light on this field. Their research team elucidated the molecular mechanism by which ImP impairs glucose tolerance and insulin signaling through the activation of the p38γ/p62/mTORC1 signaling pathway. Further studies have revealed that ImP inhibits the hypoglycemic activity of metformin via the p38γ/Akt/AMPK pathway. Structural analysis has unveiled the interaction between ImP and the adenosine triphosphate (ATP) binding pocket of p38γ. In silico analysis suggests that pirfenidone, used to treat idiopathic pulmonary fibrosis, may compete with ImP for binding to this site of p38γ, implying its potential as a candidate for combination therapy in individuals with T2D who are unresponsive to metformin. Future research should focus on elucidating the detailed mechanism of ImP binding to p38γ for structure-based drug design. While pirfenidone shows promise, clinical trials are needed to confirm its efficacy and safety in T2D patients, along with investigations into potential drug interactions.

Besides opposing the interaction involving ImP, strategies aimed at reducing ImP production, like inhibiting enzymes such as urocanate reductase, present promising new therapeutic avenues for T2D. In this regard, Venskutonytė and Koh et al. elucidated the X-ray structures of its ligand-binding domains (26), providing valuable insights for structure-based drug design and aiding in the development of inhibitors for potential treatment of metabolic disorders.

5.2 Limitations and future directions

While the existing research provides valuable insights into the role of ImP in cardiometabolic diseases, several limitations and areas for future investigation should be acknowledged. Firstly, much of the current research focuses on observational and cross-sectional studies, limiting our ability to establish causal relationships between ImP and cardiometabolic diseases. Longitudinal follow-up studies are needed to elucidate the temporal relationship between ImP levels and disease onset and progression.

Secondly, there is a noticeable lack of diversity in study populations, with a predominant inclusion of individuals of European descent in many studies. Given that the gut microbiome exhibits significant variation across geographical regions (84, 85), and considering that the production of ImP is highly dependent on bacterial activity, it becomes imperative for future studies to encompass more diverse populations. This approach will not only enhance the generalizability of findings but also allow for a comprehensive understanding of how ethnic and geographic factors may influence ImP production and its implications for cardiometabolic diseases.

Thirdly, while circulating ImP levels have been correlated with systemic inflammation in metabolic disorder (13, 18, 20), the underlying mechanisms remain unclear, highlighting the necessity for further research into ImP's regulation of inflammatory pathways. Notably, rectal administration of ImP in mice resulted in a significant increase in NF-κB, iNOS, and IL-6 expression, accompanied by a reduction in goblet cell count (23). These findings suggest ImP's potential to induce intestinal inflammation, disrupt the intestinal barrier, and alter goblet cell proliferation. Given the crucial role of intestinal barrier integrity in cardiometabolic diseases, targeting ImP to modulate intestinal barrier function holds promise for cardiometabolic disease treatment. However, additional evidence is required to substantiate ImP's regulatory role and elucidate its molecular mechanisms in this context.

Fourthly, optimal enzymatic activity of UrdA occurs under neutral pH conditions (28), while the proximal colon tends to maintain an acidic environment. Reduced production of SCFAs and increased protein fermentation in the gut contribute to the elevation of colonic pH (86), potentially facilitating ImP production. This inference partially elucidates the observed association between decreased fiber intake and increased ImP levels in certain populations, along with the correlation between a Western diet and elevated ImP content (13, 70, 71). Therefore, future research should focus on elucidating the contributions and mechanisms of factors influencing intestinal pH on ImP levels, thereby providing theoretical insights into targeting ImP-mediated pathways of cardiovascular metabolic damage. Additionally, the inhibitory effect of ImP on the hypoglycemic efficacy of metformin (36) underscores the profound impact of gut microbiota and their metabolites on drug therapy. Exploring the effects of drugs related to the treatment of cardiovascular metabolic diseases on ImP-producing bacteria and ImP content presents a novel and intriguing topic, offering potential new insights into the therapeutic mechanisms of drugs.

Furthermore, while diet has been shown to significantly influence ImP production via its effects on gut microbiota composition, it should be noted that other potential factors influencing ImP levels, such as genetic predisposition or medication use, have not yet been thoroughly investigated. Future research will be needed to explore these dimensions, as well as to further elucidate the mechanistic links between ImP and cardiometabolic diseases.

Lastly, as an increasing number of studies shed light on the correlation between gut bacteria and ImP production, as demonstrated in Table 1, it becomes evident that, like many other investigations focused on the gut microbiome, these studies raise more questions than they answer. Unveiling the precise contributions and mechanisms of these bacteria to ImP production is a vast and intricate endeavor, yet it constitutes a crucial aspect of research on ImP and warrants significant attention in future studies.

6 Conclusion

The interplay among cardiometabolic diseases underscores the importance of exploring shared metabolic pathways to enhance risk prediction and develop tailored prevention and management strategies. Over the past few years, multiple clinical studies have identified the intestinal metabolite ImP as a potential microbial signature linking insulin resistance, T2D, hypertension, obesity, NAFLD, CKD, atherosclerosis, and HF (Table 2, Figure 1). Our study emphasizes the significance of ImP as a potential biomarker and therapeutic target, highlighting the need for longitudinal research and diverse population participation to validate these associations. Future investigations should prioritize elucidating the molecular mechanisms underlying ImP's role and its contribution to the pathogenesis of cardiometabolic diseases and related comorbidities, thereby advancing treatments for these conditions.

TABLE 2 The relationship between ImP and cardiometabolic disease/risk factors.

Disease	Clinical investigation		Sample	Main results	Reference
	Country	Research object			
T2D	Netherlands	15 obese subjects (BMI > 40): 5 with, 10 without T2D	Portal and peripheral plasma	ImP levels were higher in T2D subjects than in non-diabetic subjects (portal vein: $P < 0.001$; peripheral: $P < 0.05$).	(12)
	Sweden	649 subjects, aged 50-64: 335 NGT, 119 IFG, 142 IGT, and 53 naive T2D	Peripheral plasma	Treatment-naive T2D subjects had higher ImP levels than those with normal glucose tolerance, even after adjusting for BMI, sex, and age (men: 7.6 vs. 15.1 nM; women: 11.2 vs. 24.9 nM; adjusted P < 0.0001).	
T2D	Sweden	69 subjects aged over 65 with T2D, exclusively treated with metformin	Plasma	Metformin-treated subjects with blood glucose ≥7.8 mM had higher mean ImP levels than those with <7.8 mM ($P < 0.05$).	(36)
T2D	Europe (France, Germany, and Denmark)	1958 participants: 765 T2D, 654 prediabetes, and 539 healthy controls	Serum	1. ImP levels are higher in prediabetes and T2D compared to healthy controls ($P < 0.001$). 2. High ImP quartile (Q4) is associated with increased risks of prediabetes (OR = 1.75, $P = 0.006$) and T2D (OR = 2.76, P < 0.001). 3. ImP positively correlates with HbA1c, glycemia, insulinemia, HOMA-IR, and triglyceride-glucose index, and negatively with HOMA-B ($P < 0.01$, all). 4. Higher ImP levels are linked to increased glucose ($P = 0.053$), insulin ($P = 0.02$), and C-peptide after OGTT ($P = 0.00014$), with reduced Stumvoll sensitivity index ($P = 0.004$). 5. T2D subjects have higher ImP levels (28.1 nM) than those with prediabetes (27.8 nM) or normal glucose tolerance (19.7 nM) ($P = 0.028$). 6. CVD subjects have higher ImP levels (36.7 nM vs. 25.2 nM, $P < 0.001$). 7. Serum ImP positively correlates with inflammation markers (total leucocyte count, hs-CRP, IP-10) and negatively with circulating MAIT levels ($P < 0.01$, all).	(13)
T2D and Obesity	UK	1018 females	Serum	ImP serum levels exhibit positive correlations with BMI (r = 0.18, $P = 5E-9$), fasting glucose (r = 0.065, $P = 0.05$), and visceral fat mass (r = 0.067, $P = 0.06$), and are linked to increased risk of obesity (r = 0.2, P = 8E-9) and T2D (r = 0.095, $P = 0.02$).	(15)

(Continued)

TABLE 2 Continued

Disease	Clinical investigation		Sample	Main results	Reference
	Country	Research object			
Overweight and Obesity	Netherlands	107 participants (BMI > 25, medication-free for the last 3 months): 76 insulin-resistant, 31 insulin-sensitive	Plasma	1. ImP levels significantly correlated with diastolic blood pressure (r_s = 0.285, P = 0.004) and showed borderline significance with systolic blood pressure (r_s = 0.187, adjusted P = 0.060) and LDL-cholesterol (r_s = −0.181, adjusted P = 0.064); 2. Among overweight/obese non-T2D subjects, plasma ImP concentrations do not significantly differ between insulin-sensitive and insulin-resistant individuals (16.3 nM vs. 19 nM).	(14)
T2D	China	96 T2D subjects	Plasma	ImP levels significantly correlated with postprandial blood glucose (r = 0.249, P = 0.028)	(31)
T2D, CHF, and CKD	China	260 participants: 23 NGT, 48 NGT + CHF, 83 prediabetes + CHF, 56 prediabetes + CHF + CKD, 34 T2D + CHF, and 16 T2D + CHF + CKD	Serum	1. ImP is a common metabolite in patients with prediabetes/T2D, CHF, and CKD. 2. ImP levels increase 1.1–1.6 times with each additional CHF comorbidity (e.g., NGT vs. NGT + CHF: FC = 1.1, P = 0.047; T2D + CHF vs. NGT + CHF: FC = 1.6, P = 0.055; T2D + CHF + CKD vs. T2D + CHF: FC = 1.5, P = 0.011). 3. ImP strongly associates with CKD biomarkers, including creatinine, Cystatin C, and eGFR.	(21)
CVD and HF	Europe (France, Germany, and Denmark)	1985 participants: 133 HF, 282 CVD, and 1569 without CVD/HF (metabolic disease and healthy subjects)	Serum	1. HF patients had higher ImP levels than those with CVD (P < 0.01) or non-CVD/HF (P < 0.001). 2. The highest ImP quartile increased HF risk (adjusted OR = 3.02, P < 0.05). 3. Elevated ImP levels were associated with reduced LVEF (adjusted P < 0.001). 4. ProANP and NT-proBNP levels rose with higher ImP quartiles (P < 0.001).	(51)
	USA	2155 participants: 407 HF, 1331 CVD, and 417 without CVD/HF		1. Circulating ImP levels are associated with T2D (P < 0.01); 2. CVD and HF patients have higher ImP levels than those without (P < 0.001); 3. The highest ImP quartile significantly increases HF risk (adjusted OR = 2.89, P < 0.001); 4. Elevated ImP levels are associated with reduced LVEF (adjusted P < 0.01); 5. NT-proBNP levels rise with higher ImP quartiles (adjusted P < 0.0001); 6. High ImP (Q4) predicts higher mortality risk (adjusted HR = 1.85, P < 0.01); 7. ImP outperforms traditional predictors for 5-year mortality (P < 0.01).	
CKD	USA	2438 participants: 292 CKD (Stages 1–5: 134, 76, 73, 7, and 2, respectively), 2146 without CKD.	Serum	1. Longitudinal ImP elevation is linked to reduced eGFR (Beta = −1.33, 95% CI [−2.31, −0.35], P = 0.008); 2. Elevated ImP levels predict worsening kidney traits (lower eGFR, higher UAC ratio, CKD) over ~6 years, especially in diabetics.	(17)
Atherosclerosis	USA	493 females with or at high risk of HIV infection: 84 with plaque, 236 without plaque	Plasma	1. Plasma ImP positively associates with carotid artery plaque (P = 0.043). 2. ImP positively correlates with inflammatory markers (CX3CL1, TNFSRF9, LIF-R; P < 0.05) 3. ImP-associated gut bacterial score correlates significantly with higher plasma ImP levels and increased plaque odds (OR = 1.31).	(18)

(Continued)

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TABLE 2 Continued

Disease	Clinical investigation		Sample	Main results	Reference
	Country	Research object			
Atherosclerosis	Denmark	254 HIV-infected participants: 60 obstructive CAD, 80 nonobstructive CAD, 114 without CAD	Plasma	1. Plasma ImP levels were higher in obstructive CAD patients compared to nonobstructive CAD ($P = 0.022$) and no CAD ($P = 0.00047$). 2. High ImP levels (Q4) increased obstructive CAD odds in univariable analysis (OR = 2.3, $P = 0.01$) but not after adjustment ($P > 0.05$).	(19)
Cardiometabolic health	USA	446 females: 300 HIV+, 146 HIV−	Plasma	ImP levels positively correlate with HbA1C ($P < 0.01$) and negatively with FGF-21 (rs = -0.229, $P < 0.001$).	(30)

BMI, body mass index; CAD, coronary artery disease; CHF, chronic heart failure; CKD, chronic kidney disease; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; FC, fold change; HbA1C, glycated hemoglobin; HF, heart failure; HIV, human immunodeficiency virus; HOMA-IR, homeostatic model assessment of insulin resistance; HR, hazard ratio; hs-CRP, C-reactive protein; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; ImP, imidazole propionate; IP-10, interferon gamma-induced protein 10; LDL, low-density lipoprotein; LVEF, left ventricular ejection fraction; MAIT, mucosal-associated invariant T cells; NGT, normal glucose tolerance; NT-proBNP, N-terminal pro–B-type natriuretic peptide; OGTT, oral glucose tolerance tests; OR, odds ratio; proANP, pro-atrial natriuretic peptide; r, Pearson correlation coefficient; rs, Spearman rank correlation coefficient; T2D, type 2 diabetes; UAC, urinary albumin-to-creatinine.

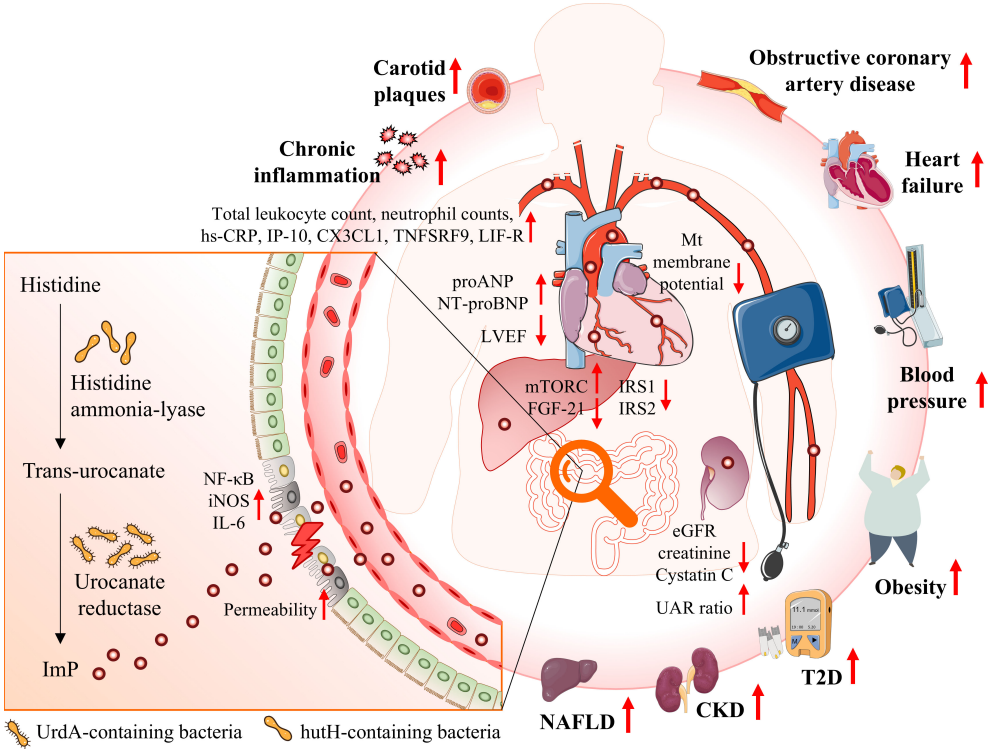

FIGURE 1
Illustrates the interplay between gut microbiota, ImP production, and cardiometabolic diseases. Epidemiological studies have identified a strong association between elevated ImP levels and an increased risk of onset of T2D and obesity, exacerbation of kidney traits in CKD, progression of atherosclerotic plaques, and elevated mortality rates in HF. ImP is a microbial metabolite derived from histidine via urocanate reductase, an enzyme encoded by the UrdA gene. This pathway is predominantly associated with certain gut bacteria. ImP may contribute to the heightened risk of cardiometabolic diseases through mechanisms such as impaired intestinal barrier function, activation of the p38γ/p62/mTORC1 signaling pathway, promoting systemic inflammation, and impairing cardiac and renal function. CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; HF, heart failure; ImP, imidazole propionate; IRS1, insulin receptor substrates 1; IRS2, insulin receptor substrates 2; MT, mitochondria; mTORC1, mechanistic target of rapamycin complex 1; NAFLD, non-alcoholic fatty liver disease; SCFAs, short-chain fatty acids; T2D, type 2 diabetes; UAC, urinary albumin-to-creatinine.

Author contributions

YZ: Data curation, Visualization, Writing – original draft, Writing – review & editing. QW: Writing – original draft. MG: Funding acquisition, Writing – original draft. FT: Visualization, Writing – review & editing. CJ: Visualization, Writing – review & editing. JC: Writing – review & editing, Validation. XT: Writing – review & editing, Validation. CZ: Writing – review & editing, Validation. YL: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. BL: Conceptualization, Supervision, Writing – review & editing. YX: Funding acquisition, Methodology, 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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Associated factors and principal pathophysiological mechanisms of type 2 diabetes mellitus

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Type 2 diabetes mellitus(T2DM) as a common chronic disease with an increasing prevalence worldwide that poses a great threat to individual health, and is characterized by chronic hyperglycemia resulting from insulin resistance (IR) coupled with β-cell dysfunction. Mitochondrial dysfunction, obesity, gut microbiota, oxidative stress and inflammation have emerged as a significant contributor to the etiology of T2DM, affecting various metabolic processes critical for glucose homeostasis. This short review underscores their role in enhancing T2DM-related molecular mechanisms and explores recent advancements in diabetic management, further highlights the importance of personalized care plans to address the complexities of the T2DM and aims to improve patient quality of life and long-term health outcome.

KEYWORDS

type 2 diabetes mellitus, pathogenic mechanism, insulin resistance, mitochondrial function, obesity, gut microbiota

1 Introduction

Type 2 diabetes mellitus (T2DM) is a renowned overgrowing endocrine metabolic disease, and occurs as a result of insulin resistance (IR) and inadequate insulin production, resulting in hyperglycemia, presenting a substantial burden on global healthcare systems (1, 2). IR is characterized by an impaired cellular response to insulin stimulation in peripheral tissues, such as the liver, skeletal muscle and adipose tissue.

As T2DM advances, progressive beta cell dysfunction occurs, results in relative insulin deficiency of insulin secretion (3). Besides that, mitochondrial dysfunction, obesity, gut microbiota, oxidative stress and inflammation are also implicated in the etiology of T2DM, these different pathogenic factors underscore the urgent need for multifactorial approach that address not only control of hyperglycemia but also the broader implications and side-effects impact of overall health and well-being (4). T2DM management, pharmacological interventions alone is insufficient, lifestyle modifications, weight management, regular physical activity and patient self-management are critical to advance diabetic care (5). In fact, by addressing multifactorial characteristics of onset and development in T2DM, a focus on individualized care and prevent the risk of complications must consider patient-

specific needs, effective and sustainable treatment preferences, comorbidities, ultimately optimizing patient overall quality of life and long-term health outcomes (6). Lastly, we discuss the latest therapeutic innovations, like gene therapy, regenerative medicine and identification of omics-related biomarkers, aid in gaining a deeper comprehension of holistic care and precision medicine among diabetic patients.

2 Mechanisms and pathogenic factors of IR

2.1 Mitochondrial dysfunction

Mitochondria, commonly known as the "powerhouses" of cells, containing their own double-stranded DNA (mtDNA), which encodes 13 polypeptides constitute mostly part of the electron transfer chain (ETC) (7). In fact, the mitochondrial ETC is the site of oxidative phosphorylation (OXPHOS), which is key in glucose metabolism and the biggest net producer of ATP in mammalian cells (8). Mitochondria play a crucial role in apoptosis, signaling, oxidation processes, and cellular energy consumption and balance, as well as are vital for the proper functioning and survival of pancreatic β-cells (9). When mitochondria are dysfunctional, uncontrolled fission or fusion occurs, like impaired mitochondrial fusion or excessive mitochondrial fission, resulting in impaired mitochondrial dynamics and mitochondrial fragmentation. Reactive oxygen species (ROS) are next to the location of the mtDNA and produced by OXPHOS, this makes the mtDNA highly susceptible to oxidative damage, thus increasing the probability of mutations and further disturbing mitochondrial energy metabolism (10, 11). Meanwhile, this oxidative damage compromises mETC function and worsens energy failure, in addition to oxidative stress and dysfunctional mitochondria, which have been implicated in the part pathogenesis of various diseases, including DM (12). Peroxisome proliferator-activated receptors (PPARs) agonists, like pioglitazone is a promising thiazolidinediones and restores mitochondrial function in patients with T2DM (13). Glimins represent a new class of oral glucose-lowering drugs, primarily targeting the mitochondrial respiratory chain complex to reduce the production of ROS and prevent mitochondrial meability transition pore opening, thereby restoring mitochondrial function in skeletal muscle, liver, and pancreas of diabetic patients (14, 15).

2.2 Mitochondrial biogenesis

Mitochondrial biogenesis is implicated in cells grow and mitochondrial responses to environmental cues and metabolic demands, which is a key feature of mitochondrial function, its dysregulation contributes to the development and progression of T2DM (16). Transcriptional coactivators peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), initiates mitochondrial biogenesis pathway by sequential activations of nuclear transcription factors, including nuclear respiratory factors (NRF1/2), estrogen-related receptor-alpha(ERR-α), followed by mitochondrial transcription factor A (TFAM) and mtDNA replication and transcription. Dysregulation of PGC-1α activity leads to impaired mitochondrial biogenesis and gene expression in oxidative phosphorylation and metabolic disorders (17). Sirtuin 1 (Sirt1) is a nicotinamide adenine dinucleotide (NAD+) -dependent histone deacetylase that plays a significant role in modulating PGC-1α activity, promoting mitochondrial biogenesis and renewal (18). Inflammatory cytokines, calcium (Ca^{2+}) regulation and metabolic stressors, such as hyperglycaemia and dyslipidaemia, are also linked to inhibit mitochondrial biogenesis and promote onset of IR (19). Substances such as 5-Aminoimidazole-4-carboxamide ribotide, GW501516, and various natural compounds present in epicatechin, have been identified as potential pharmacological via stress kinases, transcription factors and peroxisome proliferator-activated receptors (PPARs) activation, as well as mitochondrial function restoration to alleviate metabolic abnormalities (20). Besides, mitochondrial gene therapy has emerged as an innovative tool hold significant promise for restoring mitochondrial function and enhancing cellular bioenergetics (21). Addressing mitochondrial dysfunction and T2DM crosstalk is crucial for understanding the biochemistry mechanisms involved in overproduction of ROS, reduced ATP synthesis, dysregulated mitochondrial dynamics, poor mitochondrial biogenesis, and impaired mitochondrial gene expression, resulting in imbalance between energy generation and consumption, further exacerbate metabolic disorders, which may be exactly identified the relationship between mitochondrial dysfunction and molecular mechanisms underlying T2DM is multifaceted and complex (16) (Figures 1a, b).

2.3 Obesity

An long-term imbalance in energy between intake and expenditure is a key characteristic of individuals with obesity, and then chronic exposure to hyperglycemia, dyslipidemia, glucotoxicity impairs β-cell function and viability, leading to progressive deterioration of insulin secretion capacity (22, 23). Brown adipose tissue (BAT) is a distinct type of adipose tissue that dissipates energy and plays a natural anti-obesity role. White adipose tissue (WAT) primarily stores energy. Disturbances in BAT and WAT homeostasis have been associated with certain microbial imbalances and onset of obesity (24). Given that obesity has been closely implicated in the pathogenesis of T2DM (Figure 1c). Browning agents, such as cold exposure, β-3 adrenergic receptor agonists (CL 316243, BRL 26830A), short-chain fatty acids (butyrate, propionate, acetate), are compounds that can promote the conversion of WAT into BAT, may hold promise as a target for treating and preventing T2DM (25, 26). Besides, as reciprocal causation between obesity and T2DM, significant progress in the pharmacological treatment revolve around insulin resistance-mediated obesity. Metformin is the preferred first line antidiabetic drug, exerts its therapeutic effects by increasing insulin sensitivity,

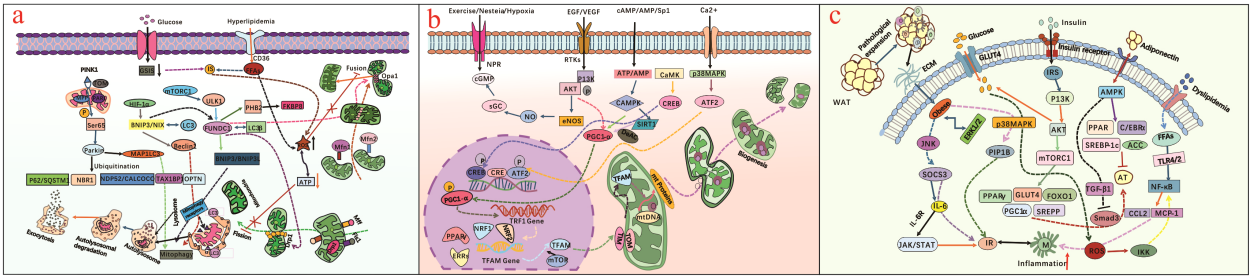

FIGURE 1
(a) illustrates mitochondrial dysfunction (abnormal mitochondrial fusion or excessive mitochondrial fission) triggers mitochondrial fragmentation, anomalous mitophagy, overproduced ROS, reduced ATP synthesis, dysregulated mitochondrial dynamics, further impairs cellular metabolism and contributes to T2DM progression; **(b)** presents the extracellular environment cues affect mitochondrial biogenesis and dynamics, as dysfunctional mitochondria fail to adapt to changing metabolic demands, leading to cellular dysfunction and T2DM; **(c)** presents adipocyte-induced obesity is significant in T2DM pathogenesis, and obesity-related dyslipidemia, inflammation and insistent hyperglycemia condition regulate IR through different molecular pathway.

glucose uptake by activating adenosine monophosphate activated protein kinase, promoting weight loss, improving lipid profiles, as well as modulating mitochondrial dynamics and biogenesis, thus contributing to its metabolic benefits in T2DM onset (27). Sodium-glucose cotransporter 2 (SGLT2) inhibitors, such as dapagliffozin and empagliffozin, have emerged as promising therapeutic agents for antihyperglycemic management (28). These substances reduce body weight, initially through a direct effect, and subsequently by shifting substrate utilization from carbohydrates to lipids, thereby reducing body fat, including visceral and subcutaneous fat (29, 30). Gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are secreted by cells in the human gut after food intake and regulate insulin release by pancreatic β-cells, and involved in blood sugar homeostasis. Tirzepatide acts on the GIPR/GLP-1R receptors, and is a recently developed drug useful in the treatment of T2DM and for weight loss, studies show its role in improving circulating levels of adiponectin, eventually influences lipid and glucose metabolism (31, 32). What needs to be emphasized that each drug has specific mechanisms and potential side effects, necessitating the significance of personalized treatment plan. Besides that, bariatric surgery like sleeve gastrectomy, one-anastomosis gastric bypass, and Roux-en-Y gastric bypass have the potential to induce remission of T2DM-related obesity (33).

2.4 Gut microbiota

The human gut microbiome is a complex ecosystem, composed of bacteria, archaea, fungi, viruses and protozoa in the human intestinal tract, which participate in material and energy metabolism, each exerting a unique influence on host metabolism (34, 35). In the context of T2DM, the gut microbiome exhibits notable changes, termed dysbiosis, which is closely related with dysregulation of a host metabolism, where there is an increase in bacteria that negatively impact metabolic health and a decrease in beneficial bacteria (36, 37). Bacteroides uniformis and Bacteroides acidifaciens can negatively improve glucose tolerance and insulin sensitivity, and are instrumental in managing T2DM, Faecalibacterium, Akkermansia, and Roseburia also exhibit similar negative correlations with the metabolic diseases (38, 39). Therefore, diabetic patients often exhibit an increase in harmful bacteria, such as Escherichia and Prevotella, Iatcu OC (40). Short-chain amino acids (SCFAs),like butyrate, propionate and acetate regulate pancreatic beta-cell activity, reduce hepatic gluconeogenesis, and modulate immune system functions (41). Moreover, the alternation of branched-chain amino acids (BCAAs) increases liver gluconeogenesis and inhibits liver adipogenesis, directly contributes to the pathogenesis of T2DM (42, 43). Notably, the changes in gut microbiota are related to LPS-induced inflammatory responses, exacerbating IR in T2DM,further emphasizing microbiome's role in glucose homeostasis and immune response (44) (Figure 2a). This emerging field offers diverse applications, particularly in modifying the gut environment through the administration of probiotics, including Bifidobacterium (adolescentis, animalis, bifidum, reuteri, breve, longum)and Lactobacillus (acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus, and salivarius) can potentially alleviate or even reverse the metabolic dysfunctions associated with T2DM (45). Additionally, diabetic medications Alpha-glucosidase inhibitors promote the growth of beneficial microbes such as Bacteroides, Lactobacillus, and Faecalibacterium, while reducing populations of potentially pathogenic bacteria like Ruminococcus and Butyricicoccus (46). GLP-1 agonists also promote the growth of SCFA-producing bacteria such as Bifidobacterium and Bacteroides, further supporting glycemic control and metabolic health (47). Sodium-glucose co-transporter type 2 inhibitors, such as sotagliflozin, impact the gut microbiome by decreasing the Firmicutes/Bacteroides ratio and enhancing fatty acid production (48). Spermidine can significantly change the composition and function of intestinal microbiota, moderately reduce the level of circulating LPS, improve metabolic endotoxemia, weaken cell apoptosis to enhance intestinal barrier function (49). Fecal

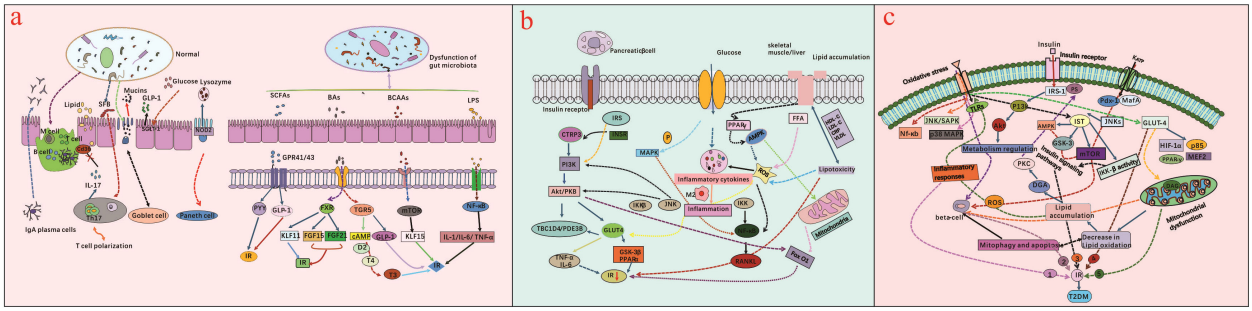

FIGURE 2
(a) illustrates dysbiosis of gut microbiota is related to LPS-induced inflammatory pathways, as well as BCAAs, SCFAs and BAs disrupt metabolic pathways, eventually triggers IR by impairing the gut mucosal barrier and increasing intestinal permeability; **(b)** presents inflammatory pathways impacting IR and possible action, and illustrates chronic hyperglycemia, oxidative stress, lipid metabolism and B-cell dysfunction can activate pro-inflammatory pathways, further exacerbate IR in T2DM; **(c)** presents oxidative stress-induced insulin resistance-related signaling pathways, and summarizes the mitochondrial dysfunction, lipid oxidation, insulin signaling pathway, inflammatory response, as well as its related pathways are instrumental in regulating T2DM.

microbiota transplantation (FMT) effectively mitigates damage to the intestinal barrier through altering the microbial structure (50).

2.5 Inflammation

T2DM patients have elevated blood glucose and free fatty acids levels, dyslipidemia, impaired insulin receptor function. Metabolic inflammation is one of markedly causatives among the metabolic derangement factors (51). Specifically, chronic hyperglycemia and hyperlipidemia are the typical features of diabetes manifestations, which inevitably lead to glucolipotoxicity and in turn alter mitochondrial function. Dysfunctional mitochondria induce non-physiological generation of ROS, exacerbate the disturbance of inflammatory microenvironmental balance between adipose and pancreatic islet tissue, inevitably forming a vicious cycle (52, 53). Along with chronic production of proinflammatory cytokines and inefficient fatty acid β-oxidation, triggering a decrease in β cell insulin secretion and an increase in IR. Chronic overload of free fatty acids and glucose trigger inflammatory pathways (AMPK and PPARγ) directly or via increased production of ROS, which is the possible causative factor in the metabolic inflammation (54, 55), depicted in Figure 2b. Adipose tissue-derived cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), promote IR by interfering with insulin signaling pathway and promoting inflammation (56). The anti-inflammatory mediators, such as visfatin, plasminogen activator inhibitor-1, blocking IL-1R, anti-IL-1β,anti-TNF-α,CCR2 antagonists and IL-6R inhibitors, have emerged as available therapeutic approach for reversing T2DM (57).

2.6 Oxidative stress

Reactive oxygen species(ROS) are byproducts of mitochondrial metabolism, as hyperglycemia advances, oxidative stress in β-cells is often driven by mitochondrial ROS, which overwhelms the body's antioxidant defenses, leading to impairment of insulin signaling pathway and metabolic dysregulation, thus plays a significant role in the pathophysiology of T2DM and its associated complications causing IR (58, 59). Most antioxidants, like SS-31 (elamipretide) and SkQ1,that reduces oxidative stress and maintains mitochondrial function offer a potential treatment strategy for T2DM and its complications (60). Additionally, ROS-mediated oxidative stress activates stress kinases and pro-inflammatory pathways, such as nuclear factor κB (NF-κB),which stimulates the release of proinflammatory cytokines and further exacerbating β-cell dysfunction and IR (1) (Figure 2c). Quercetin, curcumin, resveratrol, vitamin C and vitamin D have emerged as critical mediators in this antioxidant process, linking nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and inflammation signaling to counteract β-cell dysfunction in diabetes (61). Glutathione (GSH), a water-soluble antioxidant, plays a critical role in maintaining cellular homeostasis and mitigating oxidative stress, study shows that metformin, teneligliptin, and pioglitazone not only impacts GSH redox pathway but also preserves β-cell function (62, 63). Therapeutic strategies focusing on reducing oxidative stress, enhancing antioxidant defenses, and focusing on Mediterranean diet, such as vegetables, function and preventing diabetes progression (64). It becomes increasingly evident that therapeutic strategies focusing on targeting oxidative stress pathways and enhancing antioxidant defenses as promising therapeutic strategies to preserve β-cell function and prevent diabetic progression.

3 Conclusion and perspective

Type 2 diabetes mellitus stands as the most prevalent metabolic disease globally, characterized by intricate pathophysiological mechanism, including IR, impaired glucose homeostasis and β-cell dysfunction, genetic and environmental variables are also implicated in the etiology of T2DM. Considering multifactorial

contributors of diabetes, such as mitochondrial dysfunction, obesity and gut microbiota, mutually reinforcing each other, and creating a vicious cycle that exacerbates both insulin sensitivity and resistance, underscores the urgent need for effective T2DM management strategies that not only offer improved hypoglycemic control but also address the broader health implications (5). Retatrutide is a novel triple agonist of the glucose-dependent insulinotropic polypeptide, glucagon-like peptide 1 and glucagon receptors, distinguishes itself for its favorable glycemic control ability and its broader effects such as increasing insulin secretion, improving glucose homeostasis, and refining appetite modulation, clinical trials have demonstrated significant reductions in body weight superior to dulaglutide, tirzepatide, and even semaglutide (65–67). Dorzagliatin and Cadisegliatin are promising antidiabetic drugs activate Glucokinase (GK), which regulate glucose metabolism and enhance beta cell function in diabetic patients, although they are still in the clinical stages (68, 69).

As diabetes management continues to advance, regenerative medicine has evolved significantly, with modern therapeutic avenues and a broader focus on immunomodulatory properties and adjuvant delivery biomaterials to repair damaged tissues, like mesenchymal stem cells (70). Immune cell-derived exosomes (including engineered exosomes) may also regulate the function of immune cells by transferring miRNA and mRNA, making them a promising directions reverse metabolic disorders of T2DM (71). Although they have great clinical application prospects, there are associated hurdles about immune rejection, tumorigenesis and the precise manipulation of stem cell behaviors needed to be surmounted (70). Gene therapy differs from a glucocentric approach, utilizes lentivirus, adenovirus, adeno-associated virus (AAV), along with non-viral techniques like liposomes and naked DNA, to deliver the insulin gene to target tissues, such as CRISPR-Cas9 sequencing enables to precise modification mtDNA mutations associated with T2DM, offering potential therapeutic precision and safety for metabolic disorders (72, 73).

Omic-related fields have illuminated a path of immense promise and significant potential for revolutionizing modern therapeutic interventions. Insist hyperglycemia impacts the metabolism of glucose, lipids, amino acids, as well as gut microbiota, which all in turn impact the drug responses of individuals living with T2DM.Hence, metabolomics, lipidomics and microbiomics are applied to uncover additional biomarkers that might better predict heterogeneity observed in personalized management outcome and enhance our understanding of disease mechanisms, thus offering new opportunities for various diabetic stages and patient-specific therapeutic interventions (74). Additionally, with the complex nature of T2DM and its widespread prevalence, the integration of precision medicine into all-around care should consider a myriad of factors encompass social, demographics, phenotypic, biochemical, genetic aspects, and individual patient variability (75). Complementary and alternative medicine (CAM) therapies herbal remedies like cinnamon, fenugreek, and bitter melon, allied with mindbody therapies, including yoga, tai chi, and meditation offers holistic and patient-centered approach to alleviate disease progression, have been viewed as complementary strategy to advance T2DM care (2).Moreover, interdisciplinary research is the foreword trend to develop diabetic treatment, artificial intelligence (AI) technologies analyze genes, proteins and metabolites, provide more intelligent and precise support from early screening, diagnosis to personalized treatment and monitoring, and leveraging machine learning algorithms to assess the risk trend in metabolic diseases and empower patients in self-management (76, 77). While AI has opened new options for conquering complexity of the human insulin system, sole reliance on it for personalized treatment is far from enough, it still needs collaboration with healthcare professionals, researchers, and technical implementation experts to harness the full potential of AI in diabetic management (78).

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 authors.

Author contributions

LW: Writing – original draft. LL: Resources, Writing – review & editing. JL: Methodology, Writing – review & editing. MY: Validation, Writing – review & editing. ZH: Writing – review & editing. CS: Writing – review & editing, Investigation. RY: Investigation, 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.

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Animal studies on the modulation of differential efficacy of polyethylene glycol loxenatide by intestinal flora

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Background: Gut microbiota has demonstrated an increasingly important role in the onset and development of type 2 diabetes mellitus (T2DM), Further investigations have revealed the interactions between drugs and the gut microbiome. However, there are still gaps in research regarding the potential interactions between the gut microbiota and GLP-1 and their therapeutic response in people with T2DM. In addition, Fecal microbiota transplantation (FMT) has become a promising strategy for patients with T2DM.

Design, animals and measurements: 50 healthy male C57BL/6 mice were fed a high-fat diet in combination with STZ to establish a T2DM mouse model. 40 mice were divided into the T2DM group (n=10) and the PEX168 group (n=30). the PEX168 group was divided into two subgroups of the IE group (HbA1c ≤6. 5%, n=12) and the SE group (HbA1c >6. 5%, n=12), 12 mice in each group. Using IE mice as fecal donors and SE mice as recipients, fecal microbiota transplantation was performed between the two groups, the FMT group (given fecal bacterial suspension, n=5) and the Sham group (given equal amounts of sterile saline, n=5). The intestinal microorganisms of mice in the IE group (donor) and SE group (recipient) were also analyzed for differences. To assess the protective effect of FMT on drug efficacy and T2DM, and to explore the underlying mechanisms.

Results: After 10 weeks, compared with the control group, the HbA1c of the experimental group was significantly reduced, still, the level of HBA1c of the mice in the unsatisfactory group was significantly higher than that in the ideal group. Compared with the unsatisfactory group, fasting blood glucose, 2h postprandial blood glucose, blood glucose AUC and body weight were significantly reduced in the ideal group. 16srDNA sequencing showed that the levels of Bacteroidota, Akkermansia, Parabacteroides, Bifidobacteria and other bacteria in the ideal efficacy group were significantly higher than those in the non-ideal efficacy group ($P<0.05$). The levels of Firmicutes, Romboutsia, Clostridium, Turicibacter and other bacteria in the unsatisfactory group were significantly higher than those in the ideal group ($P<0.05$). The dominant flora of mice in the ideal drug efficacy group was negatively correlated with HbA1c and blood sugar, and the dominant flora of mice in the unsatisfactory drug efficacy group was positively correlated with pro-inflammatory factors such as blood sugar. Moreover, FMT treatment significantly improved the efficacy of PEX168 and liver steatosis in the group with unsatisfactory efficacy.

Conclusion: In summary, we used the combined method of 16S rDNA and metabolomics to systematically elucidate the efficacy of microflora on PEX168 and the possible mechanism of FMT in treating T2DM by PEX168. The difference in intestinal flora between individuals can affect the therapeutic effect of drugs. Moreover, FMT therapy can affect multiple metabolic pathways and colonization of beneficial bacteria to maintain the drug's therapeutic effect on T2DM mice.

KEYWORDS

type 2 diabetes, intestinal flora, GLP-1 receptor agonists, polyethylene glycol exenatide, fecal microbiota transplantation, 16S rDNA

Introduction

Globally, diabetes is becoming increasingly prevalent, according to the International Diabetes Federation (IDF). It is predicted that 642 million people will have diabetes by 2040 (1, 2), with T2DM comprising over 90% of these cases. T2DM is a chronic metabolic condition primarily characterized by insulin resistance (IR) and a reduction in insulin production. Once without effective treatment, it might lead to a composite of microvascular or macrovascular complications, for instance chronic kidney disease, diabetic eye disease, and cardiovascular disease (CVD); these complications often impose a significant financial and medical burden on healthcare systems globally (1, 3).

T2DM is currently treated primarily with oral hypoglycemic drugs and insulin. While thypoglycemic traditional medicines, including metformin, sulfonylureas, thiazolidinediones, α-glucosidase inhibitors, and insulin, may exert hypoglycemic effects through different mechanisms, they are prone to adverse impacts, including hypoglycemia, weight gain, severe ketonuria, and lactic acidemia (4, 5). Therefore, clinically, there is an urgent need for drugs with stable glucose-lowering effects and a low incidence of adverse effects. Drugs that target the GLP-1 receptor (GLP-1R), such as liraglutide and losenatide, have garnered significant attention due to their ability to effectively promote insulin secretion and enhancing glucose homeostasis (6, 7); these agents offer promising options for alleviating and treating diabetes mellitus and its associated complications.

Glucagon-like peptide-1 (GLP-1) is the most potent intestinal peptide for insulin secretion identified so far, and it is secreted primarily by L cells in the ileum and colon, encoded by the human glucagon gene (8). Since 1985, studies have demonstrated that GLP-1 functions as an insulinotropic agent, reducing blood glucose levels (9). Moreover, GLP-1 agonists have also been shown to suppress appetite and delay gastric emptying, thereby contributing to weight control (10). Furthermore, GLP-1 agonists have demonstrated a lower risk of hypoglycemic events compared to insulin, and some have even exhibited cardiovascular protective effects (11), As a result, GLP-1 agonists have emerged as a promising new class of drugs with significant growth potential in the treatment of diabetes. However, naturally occurring GLP-1 is hydrolyzed by dipeptidyl peptidase 4 (DPP4), resulting in a half-life of less than 5 minutes, which limits its clinical effectiveness (12). and the convenience offered by long-acting weekly preparations has dramatically simplified and enhanced long-term glycemic control for diabetes patients (11, 13, 14). PEX168 was the only GLP-1RA to enhance the therapeutic dose without increasing the risk of hypoglycemia. As a result, drugs like PEX168 have emerged as up-and-coming hypoglycemic agents.

In healthy individuals, A stable gut microbiota composition is paramount in safeguarding the gut barrier's integrity and maintaining inflammatory balance in healthy individuals. The gut stands out as a pivotal organ responsible for endogenous GLP-1 secretion. Which also serves as a prime target for the effects of exogenous GLP-1 receptor agonists (15). It plays an important in the regulating of glucose homeostasis in the body. A diverse array of microorganisms populates the guts of all postnatal animals, humans included. The human gut, harbors approximately 1014 microorganisms belonging to 500 distinct species, all residing on the intestinal epithelial barrier (16). These gut microorganisms are important and engage in diverse physiological and metabolic functions within the human body. They play a crucial role in facilitating intestinal digestion and absorption of nutrients, regulating the expression of genes about development, differentiation, angiogenesis, and energy metabolism through numerous pathways (17), and function as environmental modulators, regulating lipid metabolism and influencing the epigenetic inheritance of the host (18).

Furthermore, recent studies have revealed that specific host proteins play a pivotal role in shaping, The composition of the intestinal flora (16). Evidently, the intestinal environment is a product of the intricate interaction between the host and its intestinal microflora. Any disruption in the balance of the intestinal flora can lead to gastrointestinal diseases like diarrhea, constipation, and chronic enteritis. Additionally, such dysbiosis has been linked to a range of other health issues, including obesity, ageing, metabolic syndrome, cardiovascular disease, and diabetes

(19). A 2017 study published in (Cell Metabolism) revealed that a particular class of bacteria in the ileum can influence the glucose-lowering effects of GLP-1 (20). Some patients, however, demonstrate resistance to GLP-1 drugs, a condition known as GLP-1 resistance (14, 15, 21). The underlying mechanisms of this resistance remain incompletely understood and require further exploration. Given mild gastrointestinal adverse reactions associated with PEX168 PEX168 usage, specific subgroups of the intestinal flora might contribute to the drug's efficacy. Additionally, it has been observed that patients undergoing PEX168 treatment may experience changes in their intestinal flora, Which can manifest as symptoms such as diarrhea and vomiting. This suggests that the effectiveness of PEX168 could be influenced by individual variations in the patient's intestinal flora, pointing to a possible interaction between the drug and the intestinal flora. Furthermore, PEX168 may also induce modifications in specific subspecies of the intestinal flora.

We observe that such suboptimal GLP-1 pharmacodynamics is not exclusive to animals treated with GLP-1 receptor agonist (GLP-1RA) drugs, it can also occur with DPP4 inhibitor drugs (22). It is suggested that diabetes drugs designed based on the GLP-1 signaling pathway exhibit a certain degree of individual variability in their pharmacodynamic effects. Therefore, our research team aims to delve into the reasons for individual differences in drug efficacy by focusing on type 2 diabetic mice that respond poorly to treatment. We hypothesize that manipulating the intestinal flora through fecal microbiota transplantation could enhance the glucose-lowering effect of PEX168. Based on our hypothesis, the individual differences in gut microbiota play a crucial role in determining the efficacy of PEX168 in treating T2DM. We postulate that FMT could potentially enhance the drug's effectiveness in suboptimal individuals by modifying and reconstituting their intestinal microbiota. To validate this, we have established a mouse model of T2DM to identify mice that exhibit both ideal and suboptimal responses to PEX168. To assess whether FMT can index improve the drug's efficacy in the suboptimal responders. And elucidate the underlying mechanism involved. and further explore the potential role of the microbiotic-intestinal axis in the pathogenesis of T2DM and the mechanisms underlying drug efficacy.

Materials and methods

Materials

PEX-168 was supplied by Haosen Pharmaceutical Co. Ltd.(Jiangsu, China), Batch No.: H20190025. The specification is 0.5 ml (0.2 mg)/bottle, stored at 4~8°C. Streptozotocin (STZ): sigma product (S-0130) is stored at -20 °C and before use, it is configured into a concentration of 1% STZ solution with a citrate buffer ice bath of 0.1 mol/l (PH=4.3). The high-fat diet was purchased from Synergy Pharmaceuticals Bioengineering Co. Ltd (Jiangsu, China). Elisa and GHbA1c kits were purchased from Beinlai Biotech Co. and the blood glucose meter is from Roche in Germany.

Animal and experimental design

50 C57BL/6 male mice (4 weeks old) were purchased from the Laboratory Animal Center of Shanxi Medical University (Taiyuan, Shanxi Province, China). The mice were bred in a 12-h dark-night cycle SPF room in standard cages (5 mice/cage) at a temperature of 22 ± 1°C. All animals had ad libitum access to water and standard chow. All animals experiments were approved by the Animal Care and Use Committee of Shanxi Medical University (Approval No. DW2023020).

The General flow chart of animal experiments is shown in Figure 1. After 1 week of adaptive feeding, the mice were fed a high-fat diet (58% fat, 5.6% carbohydrates, and 16.4% proteins) and continued to feed for 8 weeks. The mice were deprived of water and injected the next day with STZ at a dose of 60 mg/kg. Random blood glucose was measured after 7 days, and mice with values ≥16.7 mmol/l indicated successful T2DM modelling, and there were 40 mice in total. All mice were randomly divided into two groups: (the T2DM group, n=10) and (the PEX168 treatment group, n=30). Mice in the experimental group were subcutaneously injected with PEX168 (200ug/kg) once a week, and mice in the control group were subcutaneously injected with an equal volume of saline for 10 weeks. These mice were monitored weekly for indicators of glucose metabolism. After 10 weeks, the mice in the drug intervention group were divided into the IE group (HbA1c ≤ 6. 5%) and the SE

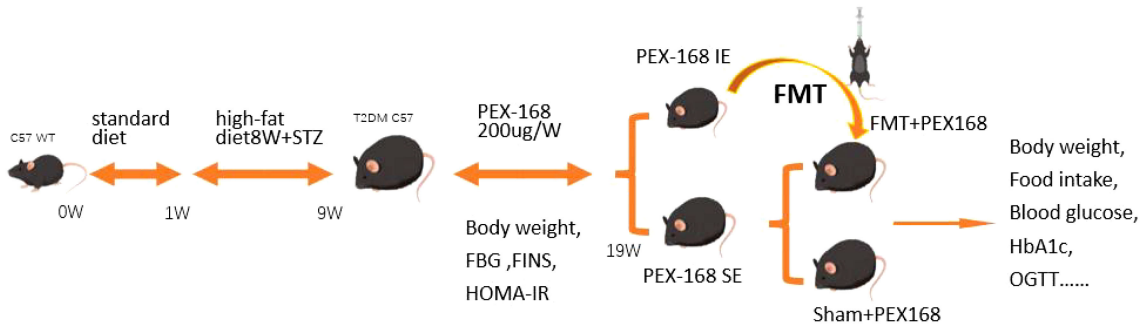

FIGURE 1
The design of the animal experiments.

group (HbA1c>6. 5%). Excluding the dead mice in the experiment, 12 remaining mice were divided into IE group and SE group according to HbA1c value. Next, We randomly divided the SE group into the FMT group(n=5) and the Sham group(n=5). FMT group mice were gavaged with 200ul of fecal bacteria solution per day, while the sham group was given sterile normal saline simultaneously. Both groups' mice were injected with PEX168 200ug/kg subcutaneously weekly for 8 weeks. The mice were given weekly measurements of glucose metabolism, such as blood glucose and body weight.

Fecal microbiota transplantation

Mice in the IE group were used as FMT donors. Collect fresh fecal pellets in the morning and freeze them immediately in liquid nitrogen. The frozen fecal samples were thawed in a constant temperature water bath at 37.5°C for 10 minutes, and then the fecal samples were suspended in sterile normal saline with a dilution ratio of 200mg feces to 2ml volume, and mixed until there were no significant large feces particles. Subsequently, the large particles in the stool are removed with a sterile 200-mesh screen, and the filtrate is then passed through a 400-mesh and 800-mesh screen to remove undigested food and more minor particulate matter. Centrifuge the suspension at 1000×g for 3 minutes to remove the insoluble material, and centrifuge the dissolved feces at 1000 g(4°C) for 3 minutes to separate the bacterial components from the residual solids. Finally, the centrifuged feces suspension is packed on a clean workbench and stored at -80°C (23) degrees for future use.

Indicators and measurements

General condition: Mice were observed every week, and their appearance, urine, feces, activity, gait, spirit, appetite, and any abnormalities were monitored.

Measurement of body weight, food intake, and blood glucose: Weight changes in mice were measured weekly. Measure the amount of food provided and record the weight of the remaining feed the next day, excluding any debris. This information is used to calculate daily food intake. The mice were not allowed to drink any water and fasted for 12 hours. The following morning, FBG levels were measured with a tail cutter and a hand-held glucose meter once a week.

Serum Biochemical Analysis: Blood was collected through the orbital vein of all mice, centrifuged at 4°C (3,000× g) for 15 min, and the supernatant was preserved. The fasting insulin (FINS) and HbA1c level were measured by mouse ELISA kit. Insulin resistance index Homa-IR=FINS x FBG/22.5 was calculated. after fasting for 12 hours. Mice were given 2 g/kg of glucose via gavage. The blood glucose levels of mice in each group were measured at 0, 0.5, 1, and 2 hours, respectively, and the changes in the oral glucose tolerance test (OGTT) were compared.

Histopathological analysis: Liver and pancreas tissues were fixed in a 4% paraformaldehyde solution and dehydrated with araffin-embedded. The embedded liver sections were stained with hematoxylin and eosin (H&E) and Oil Red O (ORO) (24). The pancreatic tissues were stained with H&E, and the pancreatic islets of GLP-1R were analyzed by immunohistochemical. Liver and pancreatic tissue images (200×) were obtained under an inverted microscope.

DNA extraction and 16S rDNA gene sequencing

The feces were collected from mice in the pharmacologically ideal and less suboptimal groups for microbiota sequencing analysis. After placing each mouse in a separate empty autoclave cage, we collected 6 to 8 fresh fecal pellets per mouse which we immediately put into sterile EP tubes. All fecal samples were frozen and stored at -80°C until further analysis (25). Following the manufacturer's instructions, We extracted microbial genomic DNA from these samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). To amplify the V3-V4 region of microbial 16S DNA we utilized paired primers consisting of a forward primer (5 ' -CCTACGGGRS gcagcag3 ') and a reverse primer (5 ' - GGACTACVVGGG TATCTAATC-3 '). The following conditions were used:95°C for 3 minutes, followed by 30 cycles at 98°C for 20 seconds, then 58°C for 15 seconds, at 72°C for 20 seconds, and finally an extension at 72°C for 5 minutes. All quantitative amplicons were pooled at uniform concentrations for Illumina MiSeq sequencing (Illumina, Inc., CA, USA). The experiments, including DNA extraction, quality assessment, library construction, and high-throughput sequencing, were performed by Jiangsu-Suichuan Biotech (Jiangsu-Suichuan, China).

Alpha diversity indices (Chao 1 and Simpson indices) were calculated using QIIME 2 (V1.9.1) (26), and differences between the 3 groups were analyzed using the Kruskal-Wallis test in R (V3.5.1). Diversity analysis was performed using weighted UniFrac distances. Subsequently, differences between groups were compared using Adonis and displayed using principal coordinate analysis (PCoA). In addition, the analysis of similarity (ANOSIM) method was used to compare differences between groups. Linear discriminant analysis (LDA) was used to compare differences in microbial abundance at the level of different taxonomic units using LDA EffectSize Tools (V1.0).

Statistical analysis

Data in this study were analyzed using R software (version 4.1.0) and SPSS software (version 26.0). And GraphPad Prism software (version 8.0.2). All data were expressed as mean ± standard deviation (SD). One-way ANOVA was used for data conforming to a normal distribution, and a t-test was used to compare the two groups. The non-parametric test (Kruskal Wallis) was used to analyze the data that does not meet the normal distribution. ns $P > 0.05$ (not significant); * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$.

Results

Changes in glucose metabolism levels in mice of IE and SE groups

There were no significant differences in preexperimental HbA1c levels among the three groups of mice, Figure 2A shows the percentage reduction in HbA1c relative to baseline in three different groups of mice after drug intervention, HbA1c was 1.25% lower than baseline in the IE group, 0.15% lower in the T2DM group, and 0.45% lower in the SE group. Compared with the SE group, HbA1c in the IE group was significantly decreased, and the difference between the two groups was statistically significant ($P < 0.001$). Both IE and SE groups had lower body weight and blood glucose than the T2DM group. Compared with the SE group, body weight and FBG in the IE group decreased significantly, and there was a difference between the two groups (Figures 2B, C, * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$). As shown in Figures 2D, E, both the T2DM and SE group mice showed severe glucose intolerance, In contrast, the glucose tolerance of mice in the IE group was significantly improved, indicating that the ability of mice in the IE group to regulate glucose damage was enhanced considerably. Serum biochemical tests showed that FINS ($P < 0.05$) and HOMA-IR ($P < 0.001$) of mice in the IE group were significantly higher than those in T2DM and SE groups, as shown in Figures 2F, G.

Long-term high-fat diet will lead to an increase in TC, total TG and LDL-C content in the body. The effect of PEX168 on serum lipids is shown in the figure. Serum TC and TG levels in T2DM mice were significantly increased; however, after PEX168 treatment, the changes of serum TG were significantly reversed ($p < 0.01$, Figures 3A, B), especially in the IE group, which significantly increased serum HDL-C level (Figure 3C) and decreased LDL-C level (Figure 3D). Effective regulation of glucose and lipid metabolism disorders in T2DM mice. (*P<0.05; **P<0.01; ***P<0.001; ***P<0.0001).

Intestinal flora analysis among each group

The fecal microbiota of three groups of mice was analyzed by 16S rDNA gene sequencing. First, an alpha-diversity analysis was performed to assess the richness and diversity of bacterial species, as shown in Figures 4A, B. The Chao 1 index ($P = 0.18$) and Shannon index ($P = 0.011$) in the IE group were higher than those in the T2DM and SE group. In addition, alpha diversity was significantly different between the IE and SE groups (Chao 1 index: $P = 0.023$; Shannon index: $P = 0.0039$; Figures 4A, B). As shown in Figure 4C, the microbial community of the T2DM group was significantly different from that of the IE and SE groups. Principal component analysis revealed that the microflora of IE group and the SE group had apparent clustering, and the microflora of the T2DM group had

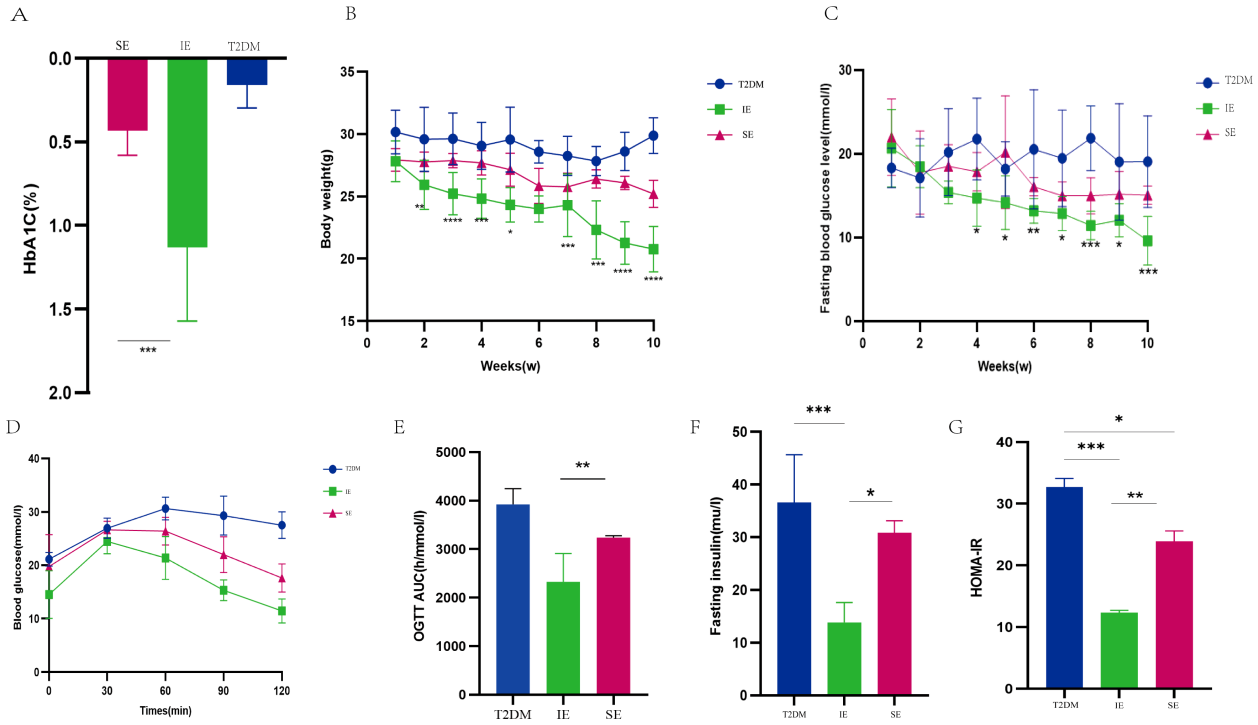

FIGURE 2
Screening and inclusion of mice with ideal and unsatisfactory efficacy of PEX168. **(A)**Baseline HbA1C level; **(B)** fasting blood glucose (FBG); **(C)** body weight (g); **(D, E)** oral glucose tolerance test (OGTT) and AUC at 12th week; **(F)** fasting insulin level; **(G)** HOMA-IR. Data are expressed as mean ± SD. Data were expressed as mean ± standard deviation (means ± s.d.) using average values. **(G)** Insulin resistance index. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). **** refers to data with P<0.001 in the statistical analysis.

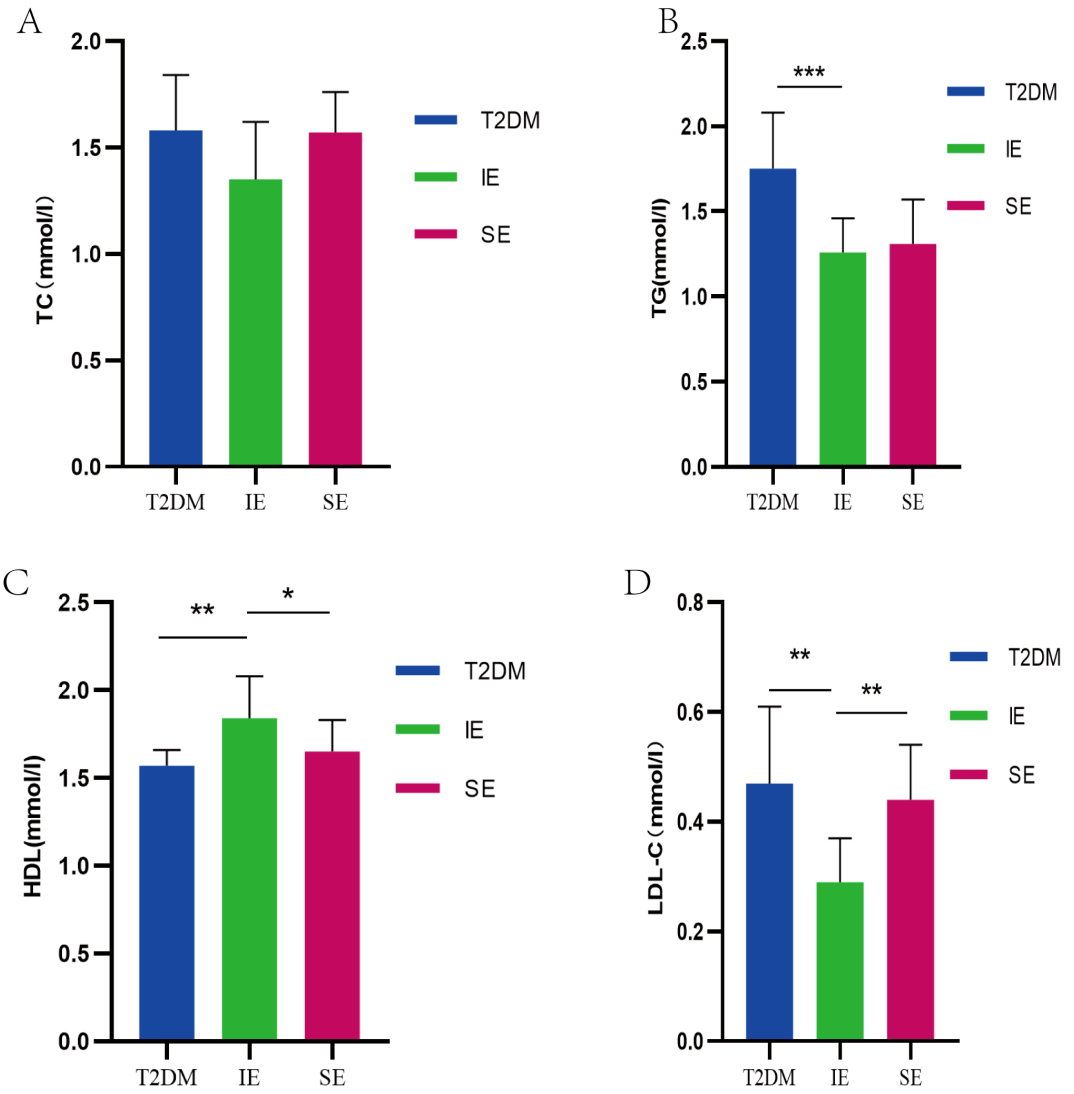

FIGURE 3
(A) TC level of mice in each group during the experiment; **(B)** TG levels of mice in each group during the experiment; **(C)** The level of HDL-C in each group of mice during the experiment; **(D)** LDL-C level of mice in each group during the experiment (*P<0.05; **P<0.01; ***P<0.001; ***P<0.0001).

significantly more flora than other groups. It is suggested that PEX168 may affect the intestinal flora structure of mice. In addition, a measure of beta diversity on weighted UniFrac distances indicated similarity in microbiota composition between groups. Adonis test and ANOISM analysis of variance (R = 0.3422, $P = 0.004$; Figure 4D) showed that there were significant differences in the bacterial flora between the three groups.

The microbial composition at the phylum and genus levels is shown in Figures 5A, B, respectively, in which a total of 30 kinds of bacterial were detected, and Firmicutes(mean value 62.87%) and Bacteroides(mean value 11.51%) were dominant in the three groups (Figure 5A). At the phylum level, the abundance of Firmicutes in the IE group was significantly lower than that in the SE group. The abundance of Patescibacteria and Proteobacteria was similar in the three groups. Compared with SE group, the abundance of Firmicutes and Bacteroidota, Campylobacterota, Desulfobacterota and Verrucomicrobiota in group IE they were increased, while the abundance of Firmicutes and Deferribacterota decreased. The F/B ratios of the T2DM group, IE, and SE groups were 10.62, 3.31, and 5.24, respectively. The F/B ratios of the IE and SE groups were much lower than that of the T2DM group, and the F/B ratio of the T2DM group was about twice that of the IE group. In addition, among the 30 genera with the highest abundance, Clostridia_UCG-014, Helicobacter were enriched in the T2DM group, Meanwhile, Akkermansia, Lachnospiraceae_NK4A136 and Colidextribacter were significantly enriched in the IE group. Allobaculum and Olsenella were significantly enriched in the SE group (Figure 5B). We further select the top 10 genera and display their details using box plots. Compared to the experiment group, the abundance of Coriobacteriacae and Enterorhabdus in the T2DM group increased, while the abundance of most other bacterial genera decreased. The comparison of bacteria at different taxa levels showed that the overall differences of most bacterial genera among the three groups were statistically significant (* $P < 0.05$, ** $P < 0.01$; Figure 5C).

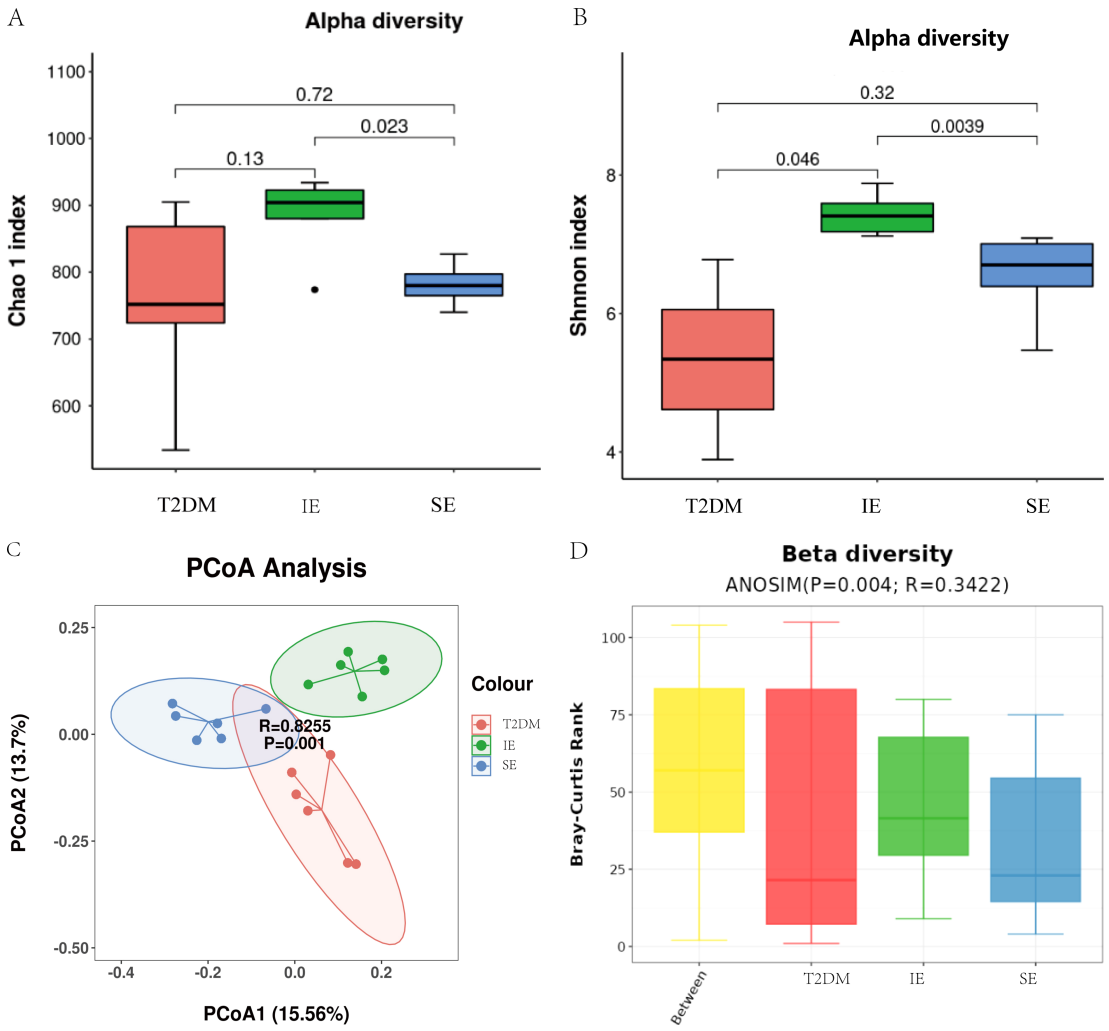

FIGURE 4
Diversity analysis of intestinal flora **(A)** Analysis of alpha diversity of gut microbiota by Chao 1 analysis. **(B)** Analysis of alpha diversity of gut microbiota by Shannon analysis. **(C)** PCoA plots of beta diversity based on weighted UniFrac analysis in different groups. **(D)** Beta diversity based on weighted UniFrac ANOSIM analysis in different groups.

Compared with the SE group, the abundance of Akkermansia, UCG_005, Rhodococcus, Staphylococcaceae, and Phascolarctobacterium showed significantly higher in IE group had increased considerably. The abundance of Romboutsia, Turicibacter, Prevotellaceae, and Lachnospira was significantly decreased (* $P < 0.05$, ** $P < 0.01$; Figure 5D).

To further identify the specific bacteria in different groups, the microbiota composition of each group was compared using linear discriminant analysis effect size (LEfSe). The results revealed the presence of a total of 48 taxa across the three groups (Figure 6A). There were distinct differences in the composition of the gut flora among the groups. Furthermore, the major taxa exhibiting significant differences among the three groups were identified by LEfSe ($P < 0.05$; LDA > 3.0). The top 3 taxa enriched in the fecal flora of the T2DM group were f_Lachnospiraceae, f_Clostridiaceae, and g_Lachnospiraceae (Figure 6B). Additionally, the bacterial taxa of the IE and SE groups were compared by the same method. The abundance of Akkermansiaceae and Verrucomicrobiota was higher in the IE group. While Peptostreptococcaceae, Romboutsia, and Turicibacter were more abundant in the SE group.

FMT treatment improvement drug treatment effect in SE group mice

There was no significant difference in baseline values between the two groups. HbA1c, FBG, OGTT, body weight and other vital indicators were carefully observed and recorded before and after transplantation. As shown in the figure, compared with the Sham group, the FMT group had significantly lower food intake (Figure 7A), body weight (Figure 7B), random glucose and FBG (Figure 7C). Additionally, the sham group exhibited severe glucose intolerance on the OGTT (Figures 7D, E). While FMT treatment significantly improved glucose tolerance in T2DM mice. Furthermore, HbA1C levels were reduced considerably in the FMT group compared to the sham group, with a decrease of

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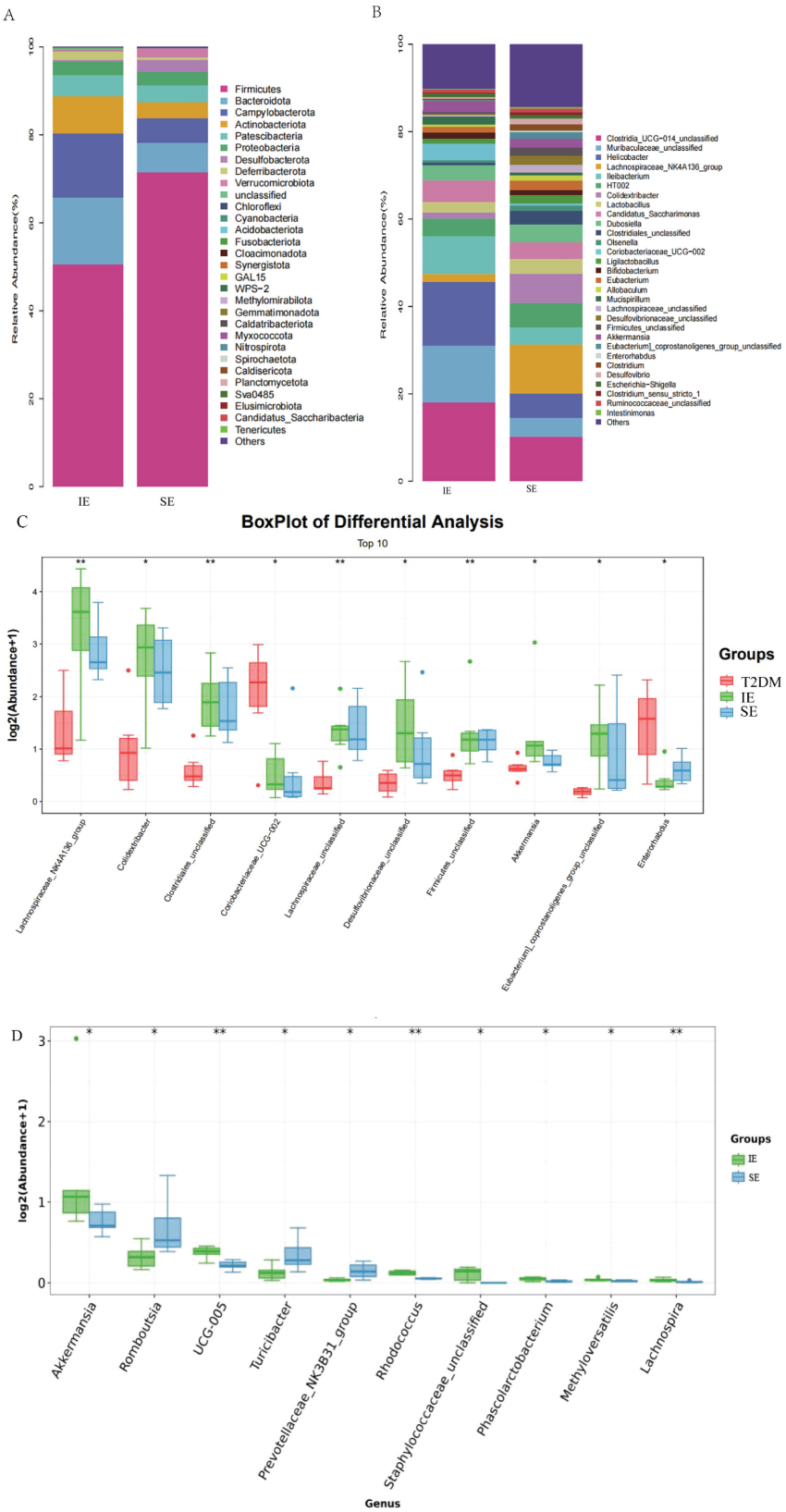

FIGURE 5
(A) The relative abundance of the gut bacterial phylum in each group. **(B)** The relative abundance of the gut bacterial genus in each group. **(C, D)** (I) Relative abundances of significantly altered bacterial genera by boxplots. Comparisons between groups were made using the Wilcoxon rank sum test (*$P < 0.05$, **$P < 0.01$).

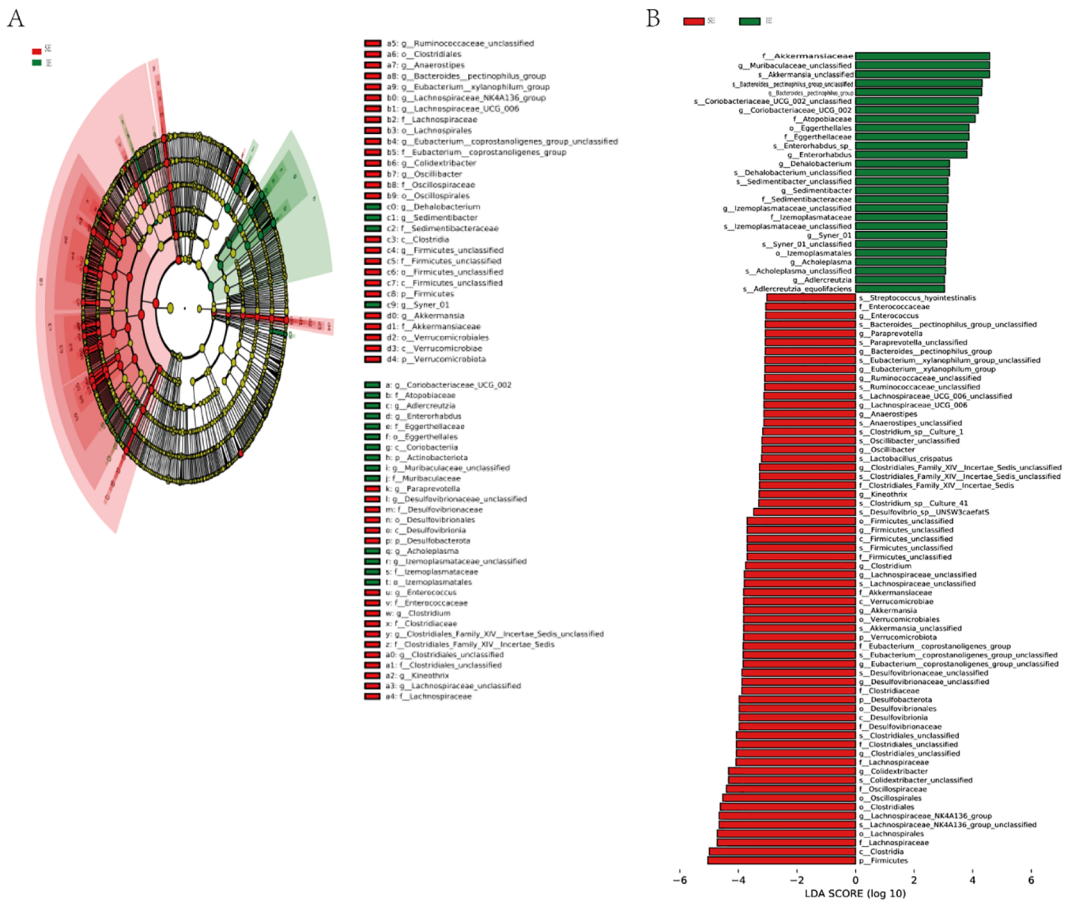

FIGURE 6
LEfSe discriminant analysis of gut microbiota in 3 groups. **(A)** Comparison of taxonomic abundances using LEfSe. The circles radiating from inside to outside represent the taxonomic levels from phyla to species. The dots located on individual circles represent different classification levels of bacteria. The size of each dot is proportional to its taxonomic abundance. The dot colors match with those of 3 experimental groups. **(B)** Histogram of linear discriminant analysis (LDA) represented significant difference in abundance of gut bacteria between each groups. A high LDA score indicates great effect of species abundance on the difference between groups.

approximately 1.4% (Figure 7F). Moreover, FMT treatment effectively reduced FINS levels in T2DM mice ($P < 0.05$; Figure 7G). Similarly, HOMA-IR was significantly reduced in the FMT group ($P < 0.001$; Figure 7H). In summary, these findings indicate that FMT treatment improves the drug efficacy of PEX168, resulting in improved glucose tolerance, reduced HbA1c levels and insulin resistance, and a protective effect against T2DM.

As shown in Figure 8, T2DM mice exhibited cytoplasmic vacuolation and hepatocyte necrosis. In addition, ORO staining is a standard experimental method to observe and analyze lipid accumulation in the liver. Oil red dye can directly react with Triglyceride (TG) in the liver to make it red, which can more intuitively observe lipid accumulation in the liver of mice. In the T2DM group, the red area was more significant, and the ORO-positive area was significantly increased, indicating that there was a large amount of lipids in the liver of mice, and steatosis was more serious. Compared with the Sham group, the vacuolar degeneration and liver injury of mice in the FMT group were significantly reduced, suggesting that FMT treatment improved liver lipid accumulation in the FMT group.

As shown in Figure 9, as observed in this study, T2DM mice had swollen islets with irregular morphology and blurring. The morphologies of the ialets of mice in the FMT group showed improvement compared to the Sham group. PEX168 is an agonist of GLP-1R, and FMT treatment can improve the drug efficacy of PEX168 and promote the synthesis and release of insulin by binding to GLP-1R. Immunohistochemical pancreatic pancreatic GLP-1R showed that PEX168 significantly increased pancreatic GLP-1R levels compared to T2DM mice and promoted insulin secretion. In addition, FMT significantly increased pancreatic GLP-1R levels in mice compared to the Sham group, can repair damaged islet cells and promote insulin secretion from pancreatic β-cells, thus exerting hypoglycemic effects.

Discussion

Many studies imply that gut microbiota dysbiosis is linked with T2DM development (17, 27). The gut microbiota is also crucial in regulating drug efficacy (28, 29). However, there are still gaps in

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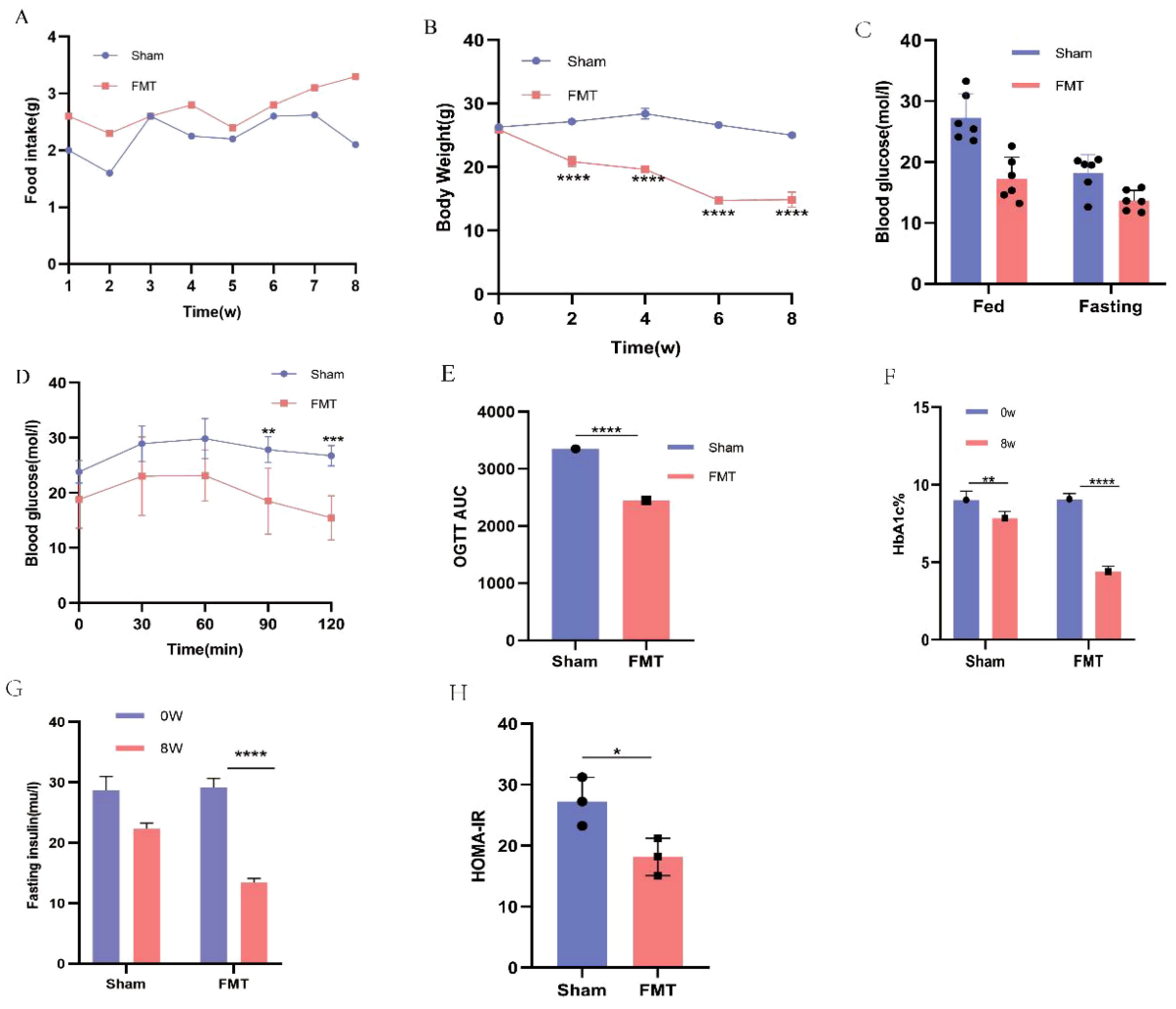

FIGURE 7
Comparison of food intake, body weight, blood glucose and serum biochemical glucose metabolism indexes between FMT group and Sham group. **(A)** body weight; **(B)** food intake; **(C)** fasting blood glucose (FBG) and Randome Blood glucose; **(D, E)** oral glucose tolerance test (OGTT) and AUC ; **(F)** HbA1C (%); **(G)** fasting insulin; **(H)** HOMA-IR;. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). **** refers to data with P<0.001 in the statistical analysis.

research on the potential interactions between the human gut microbiota and GLP-1 and their therapeutic responses in patients with T2DM. To address this, we used an STZ-induced T2DM mouse model to investigate the effect of individual differentiated gut microbiota on the efficacy of the T2DM drug PEX168. We further evaluated the protective effect of FMT treatment on T2DM to explore possible mechanisms. Our funding indicates that FMT treatment may have altered the structure of the intestinal flora in mice, which in turn affected the drug efficacy of PEX168 and improved glycolipid metabolism in mice. A specific group of gut microbiota was found to influence GLP-1 resistance (20), and many hypoglycemic drugs result in changes of intestinal microbiota. Our current study showed that PEX168 had different therapeutic effects in other individuals. Compared with the SE group, the drug intervention significantly alleviated the higher FBG levels, reduced body weight, and improved glucose tolerance and insulin resistance in the IE group. The SE group partially alleviated these symptoms and could potentially mitigate HbA1C in mice, but it could not achieve the ideal goal of drug therapy, and the impact was far less than that of the IE group mice. In addition, FMT treatment can enhance the effect of PEX168 on blood glucose and improve the glucose and lipid metabolism of SE group mice. In summary, there may be interactions between intestinal microbiota and drugs, and FMT treatment may alter the microbiota ecology, thereby improving the efficacy of drug therapy.

GLP-1RAs are novel hypoglycemic agents that have emerged in recent years and are now the focus of clinical studies for treating T2DM because they have specific outstanding advantages (30, 31). GLP-1RA can enhance glucose-dependent insulin secretion, inhibit glucagon secretion, slow stomach peristalsis, and reduce body weight by increasing satiation and decreasing appetite (32, 33). It has become a clinical research hotspot in the treatment of T2DM.

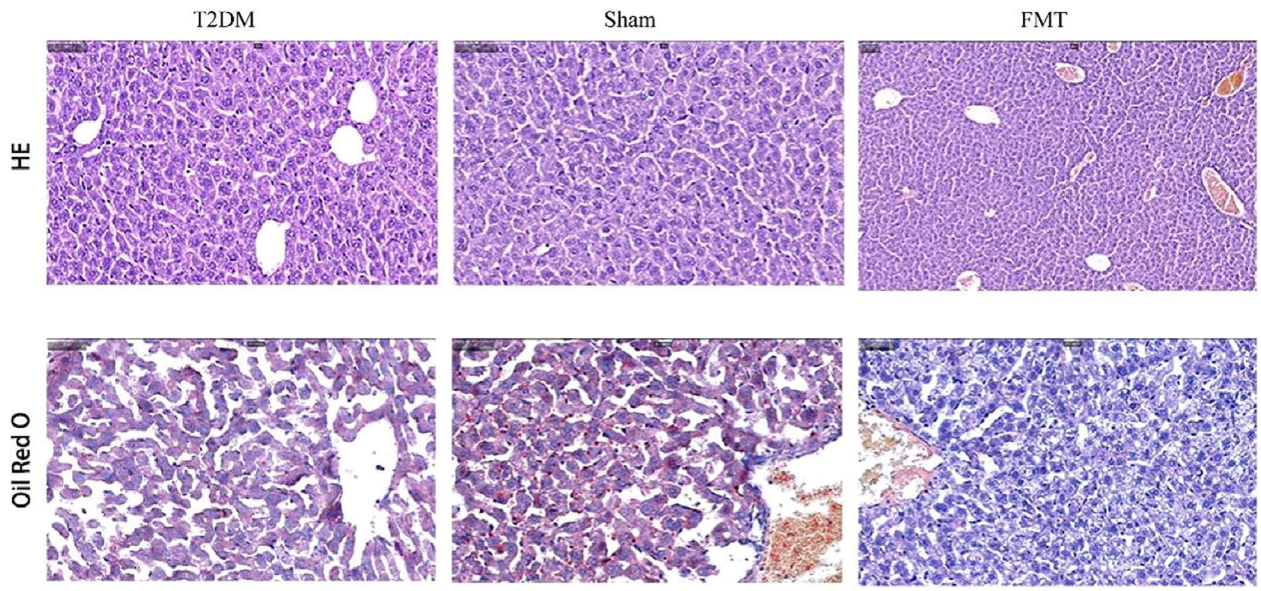

FIGURE 8
Representative images (200×) of liver tissue H&E, ORO.

Importantly, GLP-1RA have a lower risk of hypoglycemia with treatment or without sulfonylureas or insulin (34, 35). PEX-168 is the first self-development long-acting GLP-1RA in China, and It is widely used because of its advantages in reducing blood glucose levels and weight loss in patients. However, in clinical application, it has been found that not all patients achieve the desired treatment goals (25, 36). A study on reasons for GLP-1RA found that 25.0% of those who discontinued GLP-1RA reported "no improvement in quantity" as the primary reason for discontinuation, i.e., lack of glycemic improvement, lack of weight loss, etc. (36). This is consistent with our findings. Compared with mice in the IE group, the SE group failed to achieve the desired therapeutic goals of lowering blood sugar and losing weight. The biological response to GLP-1RA treatment may vary significantly depending on the individual's unique physiology characteristics.

We know that oral hypoglycemic drugs, especially those that target the intestinal system, reach the gastrointestinal tract and meet the gut microbiota. Changes in gut microbiota or functional capacity will often be observed in individuals with T2DM. For example, acarbose treatment significantly increased the abundance of intestinal bifidobacteria in T2DM patients (37). In turn, gut flora plays a vital role in regulating drug efficacy. The interaction between intestinal flora and hypoglycemic drugs is complex and bidirectional. A recent study found that GLP-1 levels can be elevated by changing the environment (38). Grasset et al. demonstrated that the gut microbial ecosystem is critical to maintaining GLP-1 sensitivity, and they found that high-carbohydrate-high-fat obese diabetes (HC-HFD) mice were severely resistant to GLP-1, compared to high-carbohydrate-no-fat (HFD) mice. Using 16S rDNA sequencing to assess the

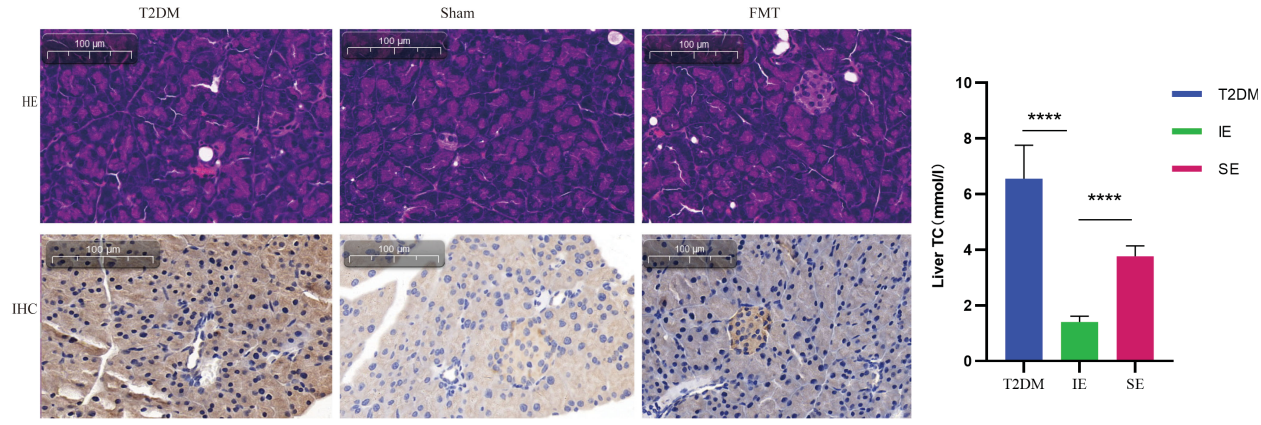

FIGURE 9
Representative images of H&E in pancreatic tissue (200×); Representative image of pancreatic islet with GLP-1R immunohistochemical staining. **** refers to data with P<0.001 in the statistical analysis.

composition of the gut microbiota, the authors further found that in the ileum of HFD mice, F/B, Lactobacillus and Clostridium significantly increased, while Bacteroidota and Defferribacterota significantly decreased. Similarly, we observed the same changes in the microbiota of mice in the SE group (20). In addition, by comparing mice lacking microbiota (germ-free mice) with mice with normal microbiota showed that GLP-1 levels were higher in germ-free mice than in conventionally reared control mice (39). They added LCMUFA, which stimulates the production of short-chain fatty acids, to the diet of mice significantly improving levels of atherosclerotic lesions and inflammatory cytokine. In turn, these benefits were consistent with improvements in the gut microbiota, i.e., lower F/B ratios, increased Akkermansia abundance, SCFA-induced GLP-1 expression and increased serum GLP-1 levels. This highlights the critical role of dysbiosis in drug efficacy.

In addition, diet and drug injections may affect the microbiome, but how and to what extent they do so may vary. First, diet is important in shaping and influencing the gut microbiome. The composition and function of the microbiomes of both humans and other mammals are profoundly affected by diet. For example, high-fiber foods can increase the diversity of gut microbes, providing rich nutrients and fiber, which is conducive to the growth of gut microbes and the maintenance of diversity (40). Conversely, diets high in fat, sugar, and salt may lead to imbalances in the gut microbiome, increasing the risk of chronic inflammation and metabolism-related diseases. On the other hand, drug injections, especially the use of antibiotics, can also have a significant impact on the gut microbiota. The diversity and abundance of intestinal flora are reduced, which increases the risk of some diseases, such as enteritis and intestinal infection (40, 41). We found that the Sham group, that is, normal saline, did not directly impact on the intestinal flora of mice, and its intestinal flora structure was relatively stable compared with before. However, the use of drugs can destroy the intestinal bacterial structure of mice to varying degrees. The diversity of the bacterial community was reduced, and the abundance of conditional pathogens such as Clotridioides was enriched, In contrast, the abundance of some butyric-producing bacteria such as Lachnoclostridium and Rhodotella, was reduced. Of course, the effects of specific drugs on the intestinal microbiota structure of T2DM mice may vary depending on factors such as drug type, dose, duration of use, and the particular condition of the mice.

We analyzed the intestinal flora composition of mice in the experimental group by 16S rDNA sequencing. The abundance of Ruminococcaeae, Lachnospiraceae, Akkermansia and Verrucomicrobiaceae in different taxa in group IE increased. Microorganisms such as Ruminococcaeae and Lachnospiraceaede have been found to promote GLP-1 release from intestinal L cells (42–44). This is consistent with our funding. The results showed that intestinal flora rapidly and significantly affected L cells and GLP-1 content. The beneficial effects of the genus Akkermansia on systemic metabolism are caused by a bacterial protein belonging to the S41A family that improves glucose homeostasis and ameliorates metabolic disorders in mice primarily by stimulating thermogenesis and GLP-1 secretion *in vivo* (45). These data suggest that Akkermansia is closely associated with the development of T2DM. These findings provide new insights into the role of Akkermansia in the pathogenesis and pharmacologic mechanisms of T2DM. It is a common genus of bacteria that produces butyric acid and propionic acid for its anti-inflammatory effects (46, 47). This is consistent with our findings. Firmicutes and Bacteroides are the central intestinal microbiota *in vivo*, and the ratio of Firmicutes/Bacteroides is negatively correlated with glucose tolerance (48, 49); other studies have demonstrated that the ratio of F/B is higher in mice with T2DM and also in mice with obesity (50, 51). In addition, FBG and FINS were negatively related to Bacteroides and Akkermansia and positively associated with Lachnospiraceae_NK4A136_group, Odoribacter and Mucispirillum (51). Proteobacteria is a relatively abundant gram-negative bacterium in T2DM mice, and its increased abundance exacerbates the inflammatory response. In conclusion, the gut microbiota is another metabolically active region that affects drug safety and efficacy. In addition, the gut microbiota may be a good target for modulating or preventing drug-induced pharmacological or toxicological effects. Since the gut microbiota has an important metabolic role, it should be further studied shortly. Moreover, the reduction in liver steatosis observed in the FMT group may be attributed to the abundant Bacteroidetes found in the fecal donors from the IE group. The latest research suggested that the Bacteroidetes genera could improve metabolic disorders and alleviate non-alcoholic hepatic steatosis by activating the Bacteroidetes-folate-liver pathway.

Given the interactions between gut microbiota and diabetes drugs, there is growing awareness that altering microbiota composition can affect metabolic phenotypes and provide a rational basis for developing personalized treatments for T2DM that target gut microbiota (52, 53). FMT has been widely used recently and has become a recognized and popular way to treat disease (54). Although the body weight and blood glucose of SE group T2DM mice decreased after PEX168 intervention, HbA1C and HOMA-IR did not improve, but the improvement of blood glucose was further prompted after FMT treatment. Many studies have shown that the gut microbiota is closely related to glucose metabolism (55), insulin resistance (54), and insulin secretion (56). There is growing evidence that the gut microbiota plays a causal role in T2DM, Which has led to targeted therapies designed to alter microbiome composition (56). FMT is a method of treating disease by rebuilding the gut microbiota (57). FMT has consistently shown the ability to overcome dysbiosis by producing profound and lasting effects on the gut microbiota, which may be a new approach to treating T2DM (58). Wang et al. found that FMT improved insulin sensitivity, increased the diversity of intestinal microbial structures, and significantly increased the abundance of butyricogenes (24). At the same time, FMT treatment successfully reduced FBG and improved glucose tolerance in diabetic mice. Restore the balance of intestinal flora and promote host homeostasis (59). In one trial, obese individuals experienced positive changes in insulin sensitivity

after receiving FMT treatment from lean, healthy individuals. Our study also found that after 8 weeks of FMT treatment, mice showed significant improvement in both peripheral insulin sensitivity and glucose tolerance. This is likely an increase in the abundance of beneficial bacteria. Our study, showed no apparent adverse reactions after FMT administration in mice; random blood glucose, fasting blood glucose, HbA1C, and HOMA-IR were all reduced, and no severe hypoglycemia occurred. FMT combined with PEX168 is superior to PEX168 alone in improving blood glucose control and insulin resistance, which provides a new direction for FMT to intervene in T2DM and FMT combined with hypoglycemic drugs to intervene in T2DM.

There are still limitations in this study remain. First, our experiments used only one animal model source, the T2DM model established by C57B/L mice, where the gut microbiota environment fed with the same diet is highly similar, and the gut microbiota may not be very different after drug intervention, assessing subtle differences or mechanisms by which the microbiota contributes to pharmacodynamic efficacy may not be sufficient. Secondly, the microbiome interacts with the drug, and the unsatisfactory effect in the drug may not be due to the difference of the microbiome, or the drug may change the microbial environment of the mouse itself, which further changes the metabolism and efficacy of the drug. Third, gavage may impact the intestinal mucosal barrier of mice, which can affect the structure of the gut microbiota and further influence the absorption and utilization of drugs. Finally, although we divided the FMT group and the Sham group and made a comparison, there was no fecal bacteria colonization analysis, and the experimental results may be biased.

Conclusion

In summary, the data suggest that in T2DM mice, the antidiabetic drug PEX168 can effectively reduce body weight, improve insulin resistance, reduce inflammatory reactions, and prevent the development of diabetes. However, individual differentiation of intestinal flora can affect the efficacy of PEX168. Further studies showed that FMT treatment improved FBG, HBA1C and HoMA-IR in T2DM mice and further improved the efficacy of PEX168, demonstrating the importance of microbiome dysregulation in the pathogenesis of T2DM. Therefore, FMT may be considered as an effective method to enhance drug efficacy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Care and Use Committee of Shanxi Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DW: Data curation, Writing – original draft, Writing – review & editing. WY: Data curation, Formal Analysis, Writing – review & editing. XJ: Data curation, Writing – review & editing. HY: Methodology, Writing – review & editing. JH: Writing – review & editing, Resources. CM: Writing – review & editing, Resources. GJ: 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.

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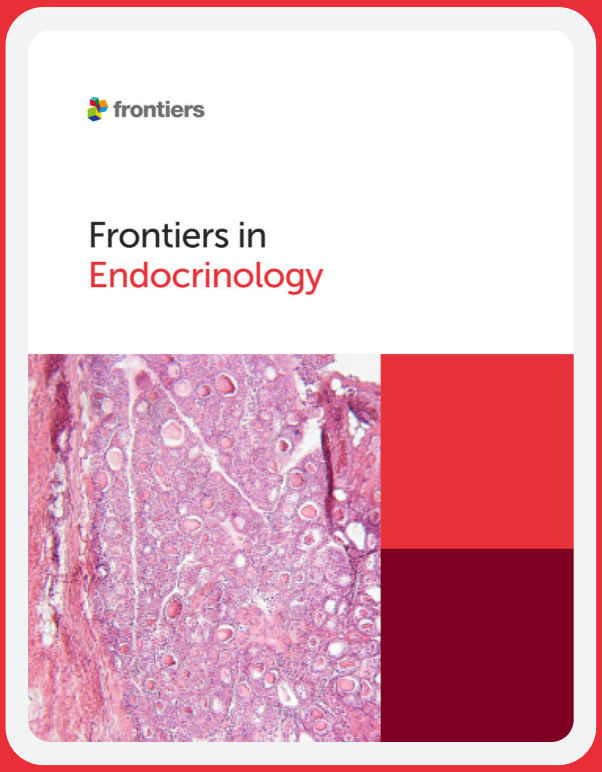

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