OXFORD

Precision Clinical Medicine, 2024, **7**: pbae003

DOI: 10.1093/pcmedi/pbae003

CLINICAL TRIAL

Double-blinded, randomized clinical trial of Gegen Qinlian decoction pinpoints *Faecalibacterium* as key gut bacteria in alleviating hyperglycemia

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Abstract

Background: Accumulating evidence suggests that metabolic disorders, including type 2 diabetes mellitus (T2DM), can be treated with traditional Chinese medicine formulas, such as the Gegen Qinlian decoction (GQD). This study elucidates the mechanisms by which gut microbes mediate the anti-diabetic effects of GQD.

Methods: We conducted a double-blind randomized clinical trial involving 120 untreated participants with T2DM. During the 12-week intervention, anthropometric measurements and diabetic traits were recorded every 4 weeks. Fecal microbiota and serum metabolites were measured before and after the intervention using 16S rDNA sequencing, liquid chromatography-mass spectrometry, and Bio-Plex panels.

Results: Anti-diabetic effects were observed in the GQD group in the human trial. Specifically, glycated hemoglobin, fasting plasma glucose, and two-hour postprandial blood glucose levels were significantly lower in the GQD group than in the placebo group. Additionally, *Faecalibacterium* was significantly enriched in the GQD group, and the short-chain fatty acid levels were higher and the serum inflammation-associated marker levels were lower in the GQD group compared to the placebo group. Moreover, *Faecalibacterium* abundance negatively correlated with the levels of serum hemoglobin, fasting plasma glucose, and pro-inflammatory cytokines. Finally, the diabetes-alleviating effect of *Faecalibacterium* was confirmed by oral administration of *Faecalibacterium prausnitzii* (DSMZ 17677) in T2DM mouse model.

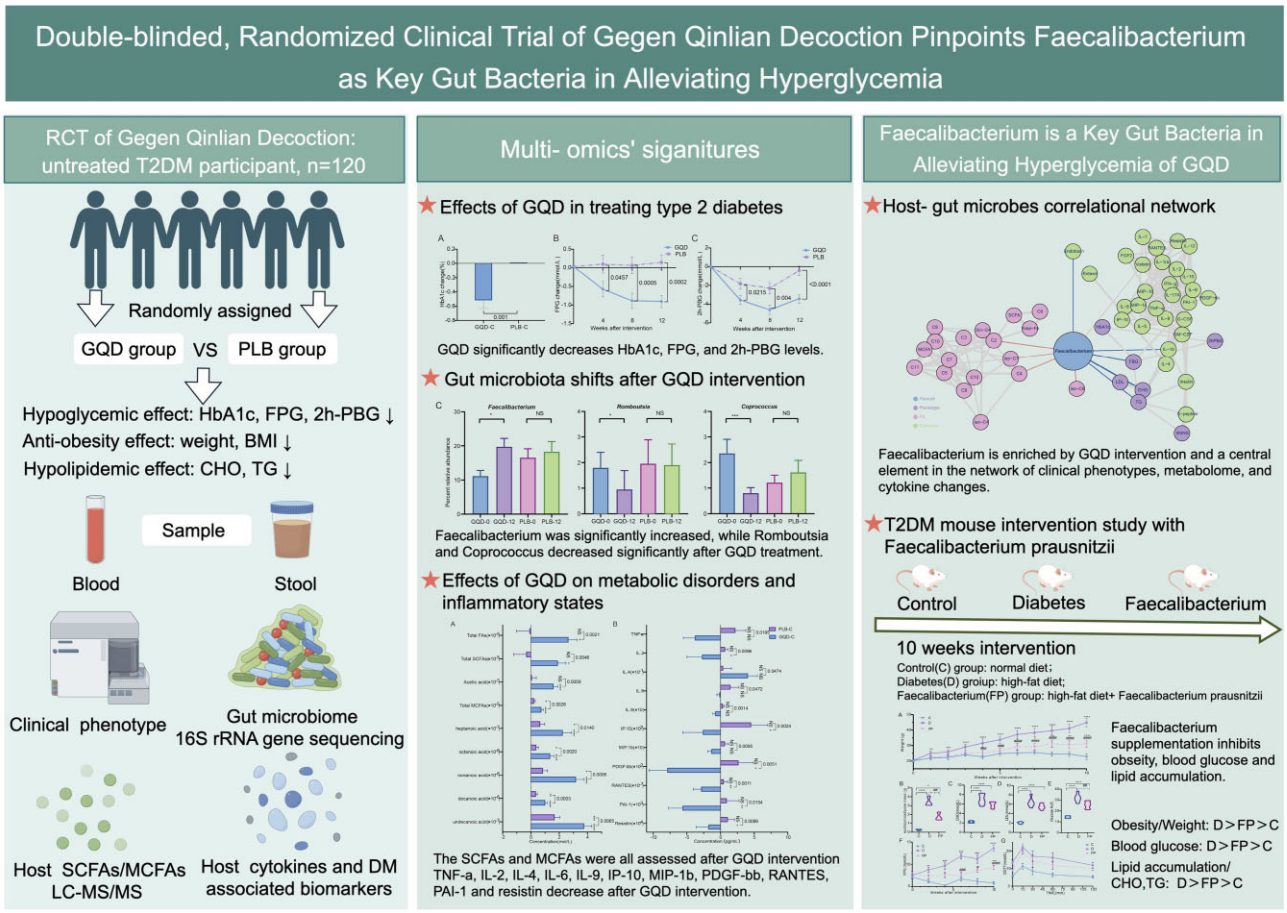
Conclusions: GQD improved type 2 diabetes primarily by modulating the abundance of *Faecalibacterium* in the gut microbiota, alleviating metabolic disorders and the inflammatory state.

Trial registration: Registry No. ChiCTR-IOR-15006626.

Keywords: double-blinded randomized controlled trial; type 2 diabetes mellitus; Gegen Qinlian decoction; *Faecalibacterium*; metabolic disorders; inflammation

Received 7 December 2023; accepted 6 February 2024. published 14 February 2024

Graphical Abstract



Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by high blood glucose levels due to insulin resistance and poor insulin secretion by pancreatic β cells [1]. The 10th edition of the International Diabetes Federation Diabetes Atlas reported that the global prevalence of diabetes in 2021 was 537 million, but the number may rise to 783 million by 2045. T2DM and its complications, such as diabetic nephropathy, diabetic peripheral neuropathy, and diabetic retinopathy, considerably burden human health and medical systems worldwide [2]. Thus, over the past few decades, T2DM has emerged at the forefront of medical research, with the focus on mechanisms and effective interventions. Recently, numerous studies have proposed that T2DM is a low-grade inflammatory disease, and its onset is associated with an immune imbalance [3].

Gut microbiota and their metabolites play key roles in the pathogenesis of T2DM by influencing the immunity of the gut mucosa and other tissues and organs involved in glucose metabolism, such as the pancreas, liver, adipose tissue, and skeletal muscle [4]. Gut microbial composition and function differ between patients with T2DM and healthy individuals, and dysbiosis of the gut microbiota influences the digestion and absorption of fats and carbohydrates, which are key components of glucose and lipid metabolic disorders. In addition, gut microbiota disturbances, such as decreased short-chain fatty acid (SCFA) and bile acid production and increased lipopolysaccharide production, aggravate systemic inflammation and cause insulin resistance [5]. For example, decreases in *Bifidobacterium*, *Faecalibacterium*, and *Akkermansia muciniphila* and increases in lipopolysaccharide-producing microbiota have been associated with inflammation and insulin resistance [6]. Furthermore, understanding how the gut microbiota affects T2DM is crucial to finding novel targets for anti-diabetic drugs, such as metformin, the most widely used drug for alleviating T2DM that is based on inhibiting gluconeogenesis. Notably, recent studies have reported that metformin modulates the composition of the gut microbiota, increasing SCFA producers such as *Akkermansia* and *Blautia* [7,8], which are closely related to reduced glucose levels.

Gegen Qinlian decoction (GQD) is a traditional Chinese medicine used to treat diarrhea and dysentery for over 2 000 years in China. A recent randomized, double-blind, placebo-controlled clinical trial (RCT) reported that GQD is an effective T2DM treatment, and an animal study demonstrated that GQD treatment shifted the intestinal microbiota [9]. Furthermore, a study of Goto–Kakizaki rats and mice with high-fat diet-induced T2DM reported an association between the anti-diabetic effects of GQD and butyrate-producing bacteria, such as *Faecalibacterium* and *A. muciniphila*. Specifically, GQD treatment significantly enriched these bacteria, which might help alleviate inflammation and insulin resistance [10]. However, the exact anti-diabetic mechanisms of GQD treatment remain unclear.

Knowing the key gut microbes affected by GQD treatment and their consequent metabolites is essential for understanding the function of GQD and for further GQD applications. Thus, we conducted an RCT, a multi-omics analysis, and an animal study to investigate the mechanisms of GQD intervention in diabetes.

Material and methods

RCT design and participants

We conducted a 12-week RCT. The study was conducted following the principles of the Declaration of Helsinki. The Ethics Committee of Guang'anmen Hospital approved this study (No. 2015EC060-02), which has been registered in the Chinese Clinical Trial Registry (registration number: ChiCTR-IOR-15006626). The inclusion criteria of T2DM patients included (i) conforming to the diagnostic criteria of T2DM (WHO,1999) and diagnosed recently without taking any hypoglycemic drugs; (ii) body mass index (BMI) $\geq$ 18 kg/m^2; (iii) not diagnosed with cancer or inflammatory bowel disease; (iv) not taking diarrhea inhibitors, laxatives, or prebiotics in the week before the first enrollment and during the entire screening; and (v) not taking antibiotics within 3 months before the first enrollment and during the entire screening. All participants provided written informed consent before sample collection.

Sample size calculation

The sample size was determined by the primary outcome hemoglobin (HbA1c). A literature study showed that the mean HbA1c reduction of the placebo group was 0.36, and the mean HbA1c reduction of the GQD group was estimated at 0.5, with the same standard deviation (SD) of 0.248 [9]. Assuming that the ratio of the GQD group to the placebo group is 1:1, using a two-sided test with a significance level (α) of 0.05 and a power (1-β) of 0.80, the required sample size is estimated by SAS v9.4 software (SAS Institute, Cary, NC, United States) to be 50 cases in each group. Allowing for a dropout of ~20%, the GQD group and placebo (PLB) group each required 60 cases, a total of 120 cases.

Randomization and masking

The study is an RCT design. With the block randomization method, the random number table is generated by an independent third party, the Institute of Clinical Foundation of the Chinese Academy of Chinese Medical Sciences, using SAS 9.4 software (SAS Institute, Cary, NC, USA). The randomized assignment sequence is placed in a sealed opaque envelope, and the blind codes are kept by the scientific research management department of the research unit and can be reproduced when needed. Study patients, the principal investigators, and study site personnel remain blinded to all patient randomization assignments throughout the duration of the study. Eligible patients are randomly allocated to either the GQD group or the placebo group according to a 1:1 ratio.

GQD and placebo instant granule preparation

The GQD formula comprised seven herbs: Gegen (Radix Puerariae lobatae), Huangqin (Radix Scutellariae), Huanglian (Rhizoma Coptidis), Gancao (Radix et Rhizoma Glycyrrhizae praeparata cum Melle), Wuweizi (Fructus Schisandrae), Zhimu (Rhizoma Anemarrhenae), and Ganjiang (Rhizoma Zingiberis). Instant GQD and placebo (PLB) granules were prepared by Jiangyin Tianjiang Pharmaceutical Co., Ltd. (Jiangsu, China; batch number 1 506 339/052 018). Briefly, the GQD herbs were mixed, boiled in water, filtered, concentrated under reduced pressure, and granulated using maltodextrin as the carrier. The phytochemical profiles of GQD have been published previously [11]. Instant PLB granules were a mixture of lactose, caramel pigment, sunset yellow pigment, tartrazine pigment, and a bitter taste agent. One GQD or PLB unit was dissolved in 150 ml of water for oral administration twice daily for 12 weeks.

Procedures

All participants were recruited from the Xicheng District and Daxing District of Beijing from 16 July 2015, to 8 December 2016; 1 798 individuals were screened. After a 2-week washout period and a series of examinations, 120 patients met the inclusion criteria and were randomly assigned to the GQD and PLB treatment groups at a 1:1 ratio for a 12-week intervention. The participants attended clinical appointments before starting the intervention (i.e. baseline, 0 weeks) and 4, 8, and 12 weeks after starting the intervention, at which time anthropometric and vital sign measurements and blood and stool sample collections were taken. The HbA1c level was the primary outcome, and the fasting plasma glucose (FPG), 2 h postprandial blood glucose (2h-PBG), body weight, and BMI were the secondary outcomes. In total, 104 participants completed the study (50 and 54 participants in the GQD and PLB groups, respectively). Finally, based on the intention-to-treat principle, 53 and 57 participants in the GQD and PLB groups were included in the full anlysis sets and safety analysis set, respectively.

Clinicians recorded all clinical data, and the Clinical Laboratory of Guang'anmen Hospital analyzed the samples for the relevant clinical phenotypes (e.g. HbA1c, FPG, insulin, fasting lipid concentrations, and hepatic and renal function indices). Blood and stool samples were collected and stored at –80 °C within 2 h of the collection. Strict screening based on clinical data integrity, preservation, and quality control of the samples resulted in 80 participants in the downstream correlation analysis (39 and 41 participants in the GQD and PLB groups, respectively). Figure 1A provides the RCT flowchart.

16S rRNA gene sequencing

Bacterial genomic DNA was extracted from frozen human stool samples using a PowerSoil DNA extraction kit (catalog no. 12888–100; Qiagen, Hilden, Germany) following the manufacturer's instructions. The V3–V4 region of the 16S RNA gene was amplified with the primer pair 341F/805R. Sequencing was performed using the Illumina HiSeq 2500 system (Illumina, San Diego, CA, USA), which produced partially overlapping 250 bp pair-end reads. Subsequent analyses were performed as described previously [10]. First, the demultiplexed FASTQ sequences were merged using the FLASH program with default parameters [12], and successfully paired sequences were processed for quality control using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). In addition, chimeras were removed by the UCHIME command of the USEARCH program and the "GOLD" database. After random rarefication of each sample to 5729 reads, composition matrices at the phylum to genus level were generated from the rarefied read table using the RDP classifier [13]. A bootstrap value threshold of 0.8 was used in the high-confidence taxonomy assignment, while the sequences with a bootstrap value < 0.8 were labeled as "unclassified". Genus-level tables were created using in-house Perl scripts.

Serum fatty acid measurements

SCFAs and medium-chain fatty acids (MCFAs) were extracted from serum using acetonitrile:water (1:1) and derivatized using 3-nitrophenylhdyrazones. SCFAs and MCFAs were analyzed using a Thermo Fisher U3000 DGLC (Waltham, MA, USA) coupled to a Sciex QTRAP 6500 Plus (Framingham, MA, USA). In brief, individual SCFAs and MCFAs were separated on a Phenomenex Kinetex C18 column (100 × 2.1 mm, 2.6 μm; Torrance, CA, USA) using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. Octanoic acid-1–13C1 purchased

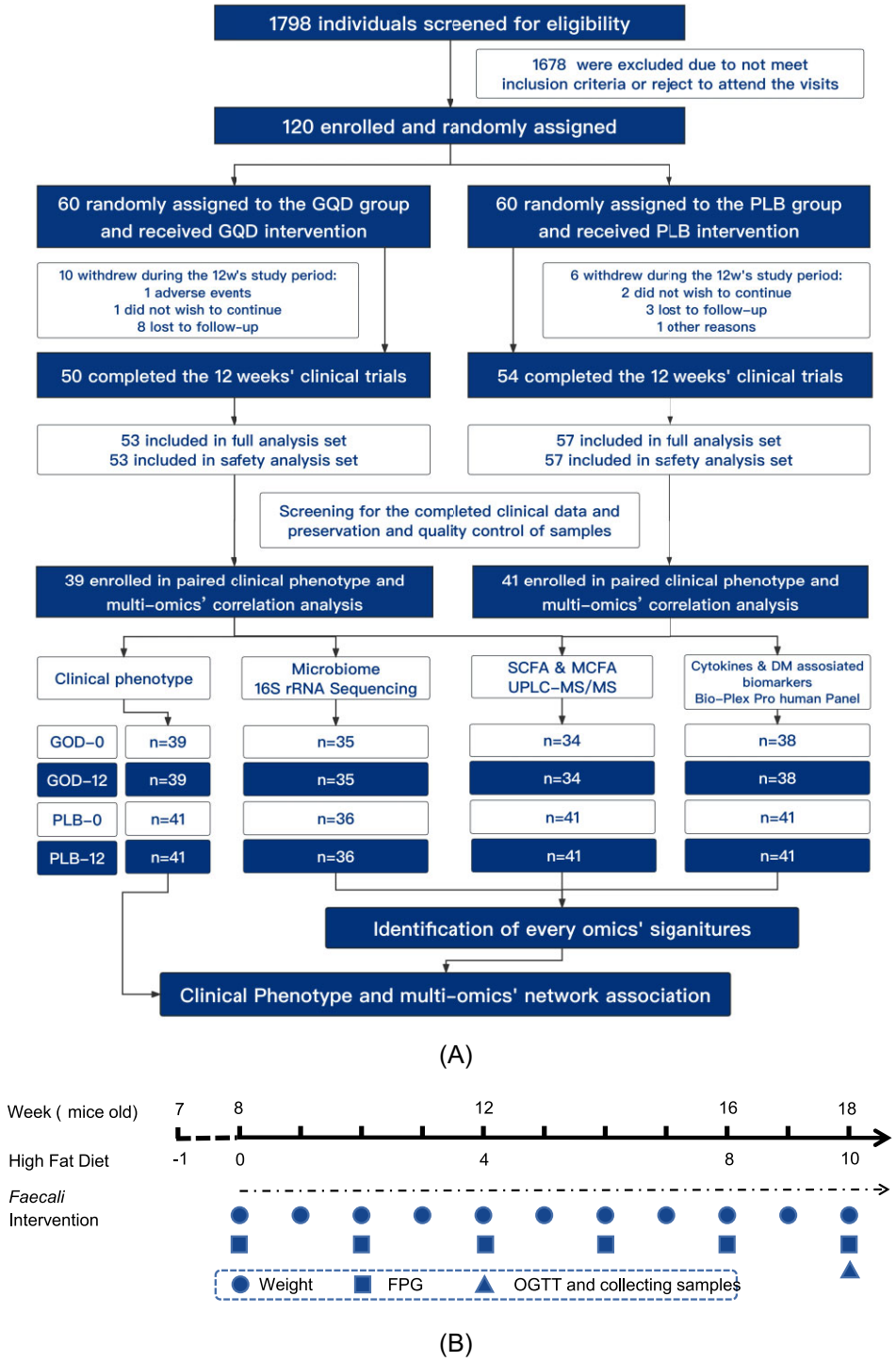


Figure 1. Study workflow. (**A**) Scheme of the RCT in humans, and (**B**) a mouse intervention study with oral administration of *Faecalibacterium prausnitzii*. DM, Diabetes mellitus; MCFA, medium-chain fatty acid; MS/MS, tandem mass spectrometry; OGTT, oral glucose tolerance test; UPLC, ultra performance liquid chromatography.

from Sigma-Aldrich (St. Louis, MO, USA) was used as an internal standard for quantitation.

Serum cytokine, diabetes-associated biomarker measurements

Cytokine concentrations [eotaxin, fibroblast growth factor 2, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor (GM-CSF)], interferon-gamma (IFN-γ), interleukin (IL)-10, IL-12, IL-13, IL-15, IL-17A, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1a, MIP-1b, platelet derived growth factor with b subunits (PDGF-bb), regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF)] and diabetes associated procoagulant

biomarkers and adipokines [C-peptide, ghrelin, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide 1 (GLP-1), glucagon, insulin, leptin, plasminogen activator inhibitor-1 (PAI-1), resistin, and visfatin] in human serum were measured using the Bio-Plex Pro Human Cytokine GrpI Panel 27-plex (Catalog No. M500KCAF0Y, Bio-Rad, Austin, TX, USA) and the Bio-Plex Pro Human Diabetes Panel 10-plex (Catalog No. 171A7001M, Bio-Rad, Austin, TX, USA). All multiplexing assays were performed using a Bio-Plex MAGPIX Multiplex reader system (Catalog No. 171 015 001; Bio-Rad) following the manufacturer's protocol. Briefly, the serum samples were incubated with capture antibody-coupled magnetic beads. After three washes using a Tecan washing station, the samples were incubated with a biotinylated detection antibody. Each captured cytokine and diabetes-associated biomarker was detected by adding streptavidin–phycoerythrin. A standard curve was used to convert the optical density values into concentrations (pg/ml). Missing values were excluded, and outliers were processed in the same way as the metabolite features before further analysis.

Microbial transplantation animal study

A total of 18 male C57BL/6J mice (18–22 g, 7 weeks old) were purchased from the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, Jiangsu, China). The care and use of animals followed the Provisions and General Recommendations of the Chinese Experimental Animals Administration Legislation, and all efforts were made to minimize animal suffering. The Ethics Committee of Guang'anmen Hospital approved this study (No. IACUC-GAMH-2021–003). Mice were housed in climate-controlled rooms (24 ± 2 °C, 60%–70% relative humidity) in a specific-pathogen-free facility with controlled 12-h light/dark cycles. All animal experimentation procedures were performed as per the Chinese Guidelines for Animal Care, which conforms to the internationally accepted use of experimental animals.

Mice were acclimatized for 1 week before beginning the study and then were randomly divided into three groups ($n = 6$): (i) control (C), conventionally raised with a normal diet (10% fat); (ii) diabetes (D), conventionally raised with high-fat diet (60% fat, D12492, Research Diets, New Brunswick, NJ, USA); and (iii) *Faecalibacterium prausnitzii* (FP), provided a high-fat diet supplemented with *F. prausnitzii* (DSMZ 17677; 5×10^{11} colony forming unit/kg of body weight/day). *Faecalibacterium prausnitzii* was administered orally by gavage in distilled water (0.1 ml/10 g of body weight) once daily for 10 weeks. The control and diabetes groups were administered equal volumes of distilled water to minimize the effects of the gavage procedure.

During the 10-week treatment, body weight was measured once per week, and FPG was monitored every 2 weeks. After 10 weeks, all animals were weighed and underwent oral glucose tolerance tests. Blood glucose levels were measured before the glucose challenge (i.e. fasting glucose, 0 min) and 15, 30, 60, and 120 min after glucose administration using an ACCU-CHEK Performa OneTouch glucometer (Catalog No. 06 454 011; Roche, Mannheim, Germany). After a 12-h fasting period, they were anesthetized with diethyl ether, and serum and epididymal adipose tissue were immediately collected and analyzed. The serum was centrifuged and separated from ophthalmic artery blood for cholesterol (CHO) and low-density lipoprotein cholesterol (LDL-C) measurements, and epididymal adipose tissue was weighed. Figure 1B provides the animal study flowchart.

Statistical analyses

Changes in clinical phenotypes, serum metabolites (fatty acids), cytokines, diabetes-associated procoagulant biomarkers, and adipokines after the 12-week GQD and PLB interventions were calculated and compared using the Mann–Whitney U test or unpaired t-test depending on the distribution. In addition, the Wilcoxon signed-rank test and paired t-test were used to compare the differences in clinical phenotype, serum metabolite cytokines, diabetes-associated procoagulant biomarkers, and adipokines before and after treatment. Statistical significance was set at $P < 0.05$.

Bayesian analyses and priors: Changes in clinical phenotypes such as weight, BMI, waistline, hipline, FPG, 2h-PBG, homeostatic model assessment for insulin resistance (HOMA-IR), HOMA-β, CHO, triglyceride (TG), low-density lipoprotein cholesterol (HDL-C), and LDL-C after 12 weeks of intervention with GQD and PLB, were all included in Bayesian analysis to estimate the average treatment effect and further validate the above clinical efficacy evaluation in another statistical analysis method. Statistical analysis was performed using Bayesian linear regression. We used Stan's default dynamic Hamiltonian Monte Carlo sampler with 4 chains with 5000 warm-up iterations and 15 000 post-warm-up iterations for each chain, and required bulk/tail effective sample sizes (ESS) of 10 000 for the parameter of primary interest (the treatment effect). We assessed convergence using the updated Rhat statistic, which we required to be ≤ 1.01 for all parameters. The result will provide the mean and two-sided 95% confidence intervals (CIs) of the posterior distribution and generate a density plot of the posterior distribution. All model diagnostics were generally adequate; bulk/tail ESS were > 40 000 for the treatment effect in all models, and the Rhat statistics for all parameters are ≤ 1.01. We used weakly informative priors centred on neutral effects. We use a normal distribution centered on 0 with a variance of 5 as the prior distribution for all parameters. Bayesian analyses were created using the *brms*, *bayesplot*, *and ggplot2* R packages (R Core Team, Vienna, Austria).

For 16S rRNA sequencing, outliers and unpaired samples were removed, and the reads were processed into equal sequencing depths. Alpha diversity and principal coordinate analyses were performed using the vegan R package. Differences in alpha diversity were analyzed using the paired t-test, while differences in the relative abundances of typical genera before and after treatment were compared using the Wilcoxon signed-rank test with GraphPad Prism v8.0 (GraphPad Inc. San Diego, CA, USA).

Samples from the GQD-0 (i.e. baseline) and GQD-12 (12-week) groups were analyzed using Spearman's correlation analyses between the relative abundance of *Faecalibacterium* and clinical traits, as well as serum metabolites. In the complete correlation network involving all variables, q-values estimated by *fdrtool* < 0.1 were considered statistically significant. Figures were created using the *ggplot2*, *ggpubr*, *pheatmap*, and *igraph* R packages (R Core Team, Vienna, Austria).

For the microbial transplantation animal experiments, one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to compare the differences in body weight, epididymal adipose mass, CHO, LDL-C, FPG, and areas under the curve among the three groups.

Data and resource availability

Raw sequence data from this study have been deposited in the Genome Sequence Archive under the accession number

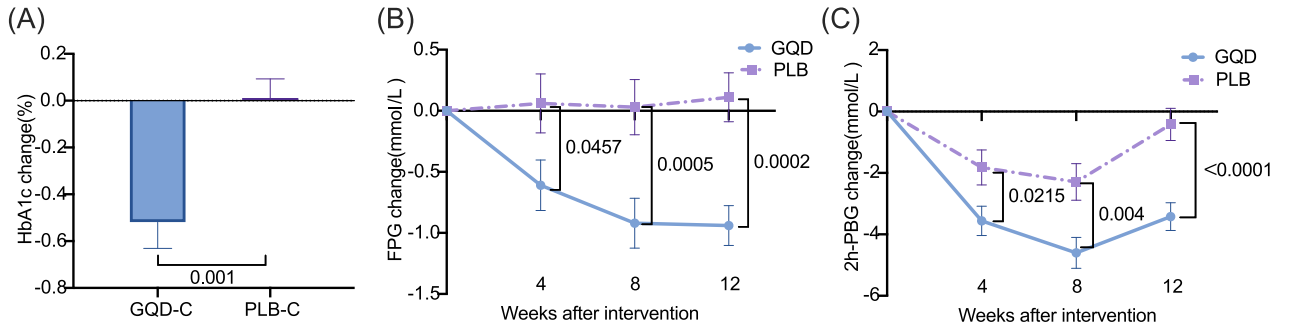


Figure 2. Hypoglycemic effects of GQD intervention. (**A**) Changes in the HbA1c level after 12 weeks of intervention with GQD or PLB (Mann–Whitney U test). Changes in the (**B**) FPG and (**C**) 2h-PBG levels after 4, 8, and 12 weeks of GQD or PLB intervention compared to the baseline values. Mann–Whitney U test: FPG (all timepoints) and 2h-PBG (4 weeks); unpaired t-test: 2h-PBG (8 and 12 weeks). *P*-values < 0.05 indicate significant differences. GQD-C: Change from baseline to 12 weeks in the GQD group; PLB-C: change from baseline to 12 weeks in the PLB group.

PRJCA007557 and website is https://ngdc.cncb.ac.cn/gsa/browse/CRA005608. All other data are available from the corresponding author upon request.

Results

GQD elicits anti-diabetic effects in humans

In the RCT, the baseline demographic and patient characteristics were similar between the GQD and PLB groups, including age ($P = 0.4613$), BMI ($P = 0.4245$), HbA1c level ($P = 0.1589$), and FPG ($P = 0.3338$) (Mann–Whitney U tests; Table S1, see online supplementary material).

After 12 weeks, the HbA1c level decreased by 0.52% [standard deviation (SD): 0.73] in the GQD group but increased by 0.01% (SD: 0.60) in the PLB group; the difference between the groups was significant ($P = 0.001$, Mann–Whitney U test; Fig. 2A). Furthermore, compared to the baseline values, the FPG level decreased after 4, 8, and 12 weeks in the GQD group [mean change: 0.61 mmol/l (SD: 1.53), 0.97 mmol/l (SD: 1.44), and 0.94 mmol/l (SD: 1.10), respectively]. The FPG also decreased after 4, 8, and 12 weeks in the PLB group but to a lesser extent [mean change: 0.10 mmol/l (SD: 1.46), 0.04 mmol/l (SD: 1.55), and 0.11 mmol/l (SD: 1.45); the differences between the two groups were significant ($P < 0.05$, Mann–Whitney U test; Fig. 2B). Finally, the 2h-PBG levels decreased during the study period in both groups, but the levels in the GQD group changed to a greater extent than those in the PLB group; the differences between the two groups were significant [mean change at 12 weeks: GQD: 3.42 mmol/l (SD: 3.05), PLB: 0.42 mmol/l (SD 3.76); $P < 0.0001$, unpaired t-test; Fig. 2C). GQD treatment also showed potential anti-obesity and anti-hyperlipidemic effects based on lower weight, BMI, HOMA-IR, and LDL-C values (Fig. S1a–e, see online supplementary material).

To further evaluate and validate the efficacy of GQD intervention, we used Bayesian analysis to compare the 12-week intervention changes in primary and secondary outcomes, including HbA1c, FPG, 2h-PBG, HOMA-IR, weight, BMI, etc. For HbA1c, the absolute difference was 0.53% (95% CI 0.25% to 0.80%). It indicated that the probability of benefit exceeding 0.25% in HbA1c was > 97.5% (Fig. 3A; Table S2, see online supplementary material). The absolute differences of FPG, 2h-PBG, and HOMA-IR were 1.05 (95% CI 0.52 to 1.58) mmol/l, 2.94 (95% CI 1.56 to 4.31) mmol/l, and 1.23 (95% CI 0.19 to 2.27), respectively (Fig. 3B–D, Table S2). As a whole, for HbA1c, FPG, and 2h-PBG, the probabilities of clinical hypoglycemic benefit with GQD were >99% when compared to placebo. The absolute difference of weight and BMI were 1.44 (95% CI 0.02 to 2.84) and 0.64 (95% CI 0.00 to1.28), which indicated that the probability of clinical weight-loss benefit with GQD was ≥97.5% compared to placebo (Fig. 3E and F; Table S2).

Five serious adverse events were reported in all participants, which included one hypoglycemic event and four gastrointestinal events. All serious adverse events were resolved with appropriate management. One hypoglycemic event was reported in the GQD group, and four gastrointestinal events were documented, two of which in GQD group and the other two in PLB group. Overall, there was no significant difference in the incidence of adverse events between the GQD and PLB groups ($\chi 2$ or Fisher's exact test; Table S3, see online supplementary material).

GQD treatment significantly alters the gut microbiota composition

To investigate the potential mechanisms underlying hyperglycemic control after GQD treatment, we investigated the fecal microbiomes of participants in the GQD and PLB groups before and after intervention using 16S rRNA gene sequencing. The alpha diversity analysis using the Shannon index showed a significant decrease in community diversity after the GQD intervention ($P = 0.039$, paired t-test; Fig. S2a, see online supplementary material), consistent with the results of our previous animal experiments [10]. The beta diversity analysis also identified significant differences in the gut microbiota composition ($R = 0.06825$, $P = 0.001$, analysis of similarities) among the groups (baseline GQD and PLB; 12-week GQD and PLB) based on the Bray–Curtis distance (Fig. 4A). Specifically, the gut microbial composition of the GQD group showed a distinct separation between weeks 0 and 12, which shifted along the major axis of the principal coordinate analysis, with PCoA Axis 1 explaining 32.06% of the variation (Fig. 4A). Among the top 10 abundant genera in all samples, the abundances of three significantly changed after GQD intervention: *Faecalibacterium* significantly increased ($P = 0.0153$, Wilcoxon signed-rank test) and *Romboutsia* ($P = 0.0208$, Wilcoxon signed-rank test) and *Coprococcus* ($P = 0.0005$, Wilcoxon signed-rank test) significantly decreased after GQD treatment (Fig. 4B, C). Changes were not observed between 0 and 12 weeks in the PLB group.

GQD treatment increases serum fatty acid levels

Faecalibacterium is a known producer of fatty acids; thus, we assessed various serum fatty acid concentrations via a targeted metabolomic analysis. GQD treatment increased the serum SCFA and MCFA concentrations after 12 weeks compared to the

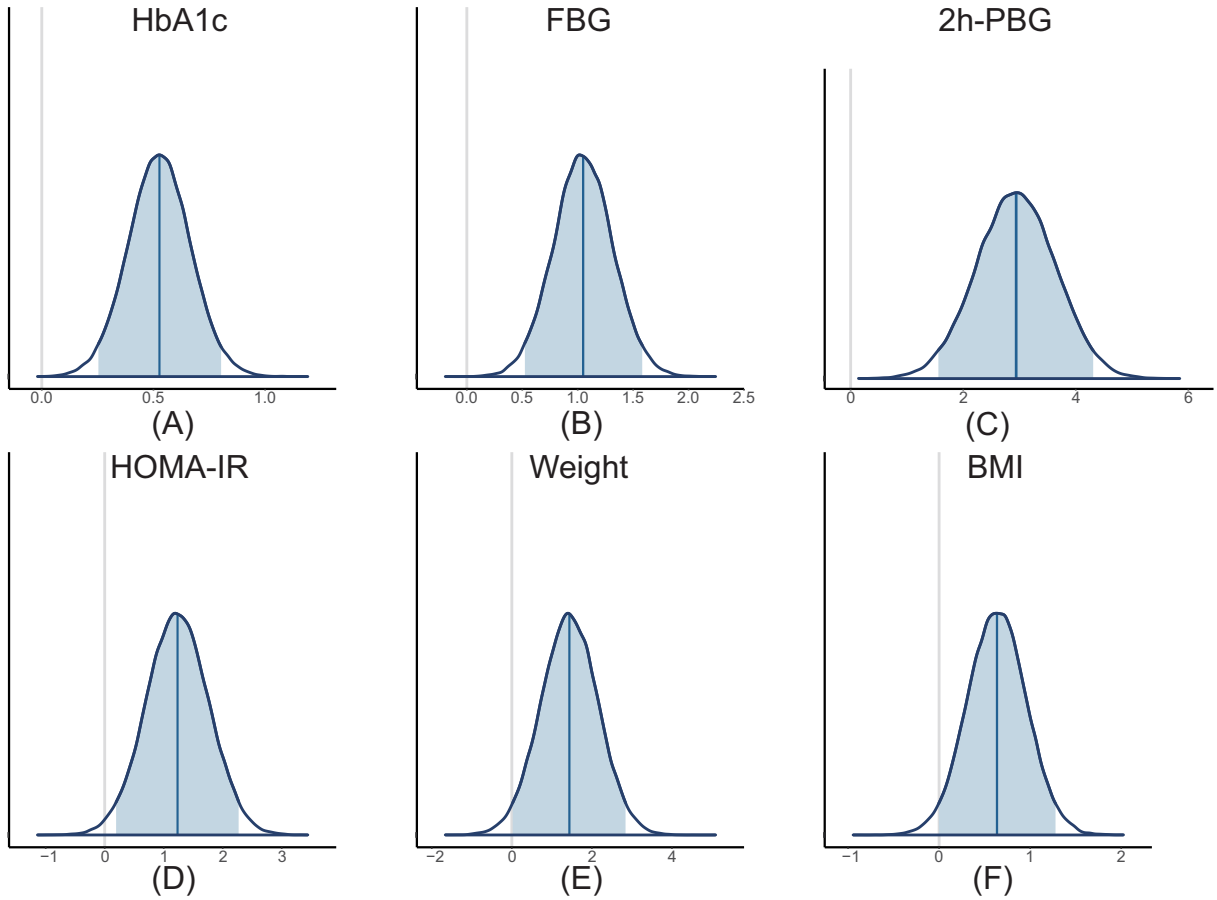


Figure 3. Bayesian analysis of the primary and secondary outcomes (using weakly informative priors). (**A**–**F**) the absolute difference and full posterior probability distributions of changes in HbA1c, FPG, 2h-PBG, HOMA-IR, weight, and BMI after 12 weeks of intervention with GQD and PLB. The vertical lines indicate the mean values (used as the point estimate) and the areas highlighted in blue indicate the percentile-based 95% credible intervals.

baseline values. The mean total SCFA concentration increased by 32.45% ($P = 0.0007$, Wilcoxon signed-rank test; Fig. S3a and Table S4, see online supplementary material), including significantly higher concentrations of acetic acid, propionic acid, and 2-methyl butyric acid ($P < 0.01$, Wilcoxon signed-rank test; Fig. S3a and Table S4). Of them, the acetic acid concentration increased the most after the GQD intervention compared to the PLB group ($P = 0.0009$, Mann–Whitney U test; Fig. 5A and Table S4).

GQD treatment also increased the mean total MCFA by 142.63% ($P < 0.0001$, Wilcoxon signed-rank test; Fig. S3a and Table S4), significantly differing from the PLB group ($P = 0.0026$, Mann–Whitney U test, Fig. 5A). Heptanoic acid ($P = 0.0140$), octanoic acid ($P = 0.0020$), nonanoic acid ($P = 0.0006$), decanoic acid ($P = 0.0003$), and undecanoic acid ($P = 0.0065$) (all Mann–Whitney U tests) were significantly enriched after GQD treatment compared to the PLB group (Fig. 5A and Table S4). These results suggest that GQD treatment prompted an overall increase in SCFAs and MCFAs in the serum of patients with T2DM.

GQD treatment alleviates inflammation, coagulation, and adipokines disorders

To further verify the overall impact of GQD on systemic inflammation and insulin resistance in patients with T2DM, we conducted a targeted analysis of serum cytokines, diabetes-associated procoagulant biomarkers, and adipokines (Fig. 5B, and Fig. S4, Fig. S5, Table S5, and Table S6, see online supplementary material). GQD treatment systematically alleviated systemic inflammation by reducing the levels of several pro-inflammatory cytokines. TNF-α ($P = 0.0195$), IL-2 ($P = 0.0096$), IL-6 ($P = 0.0472$), IL-9 ($P = 0.0014$), IP-10 ($P = 0.0024$), MIP-1b ($P = 0.0095$), PDGF-bb ($P = 0.0051$), and RANTES ($P = 0.0011$) levels were significantly lower in the GQD group than in the PLB group after 12 weeks (all Mann–Whitney U test; Fig. 5B and Table S5). Furthermore, IL-4, a typical anti-inflammatory cytokine, significantly increased after GQD treatment compared to PLB treatment ($P = 0.0474$, unpaired t-test; Fig. 5B and Table S5). Regarding diabetes-associated procoagulant biomarkers and adipokines, PAI-1 ($P = 0.0154$, Mann–Whitney U test) and resistin ($P = 0.0089$, Mann–Whitney U test) levels were also significantly lower in the GQD group than in the PLB group (Fig. 5B and Table S6). These results indicate that GQD significantly alleviated systemic inflammation in patients with T2DM.

Faecalibacterium is central to the gut microbiota in the correlational network with metabolites and cytokines

Next, we established a correlational network between clinical phenotypes, the gut microbiome, metabolites, cytokines, diabetes-associated procoagulant biomarkers, and adipokines in GQD-treated patients from weeks 0 and 12 to identify the central bacterial species contributing to the effects of GQD (Fig. 6). In the network, *Faecalibacterium*, which was enriched by GQD intervention, had the highest number of significant negative correlations with

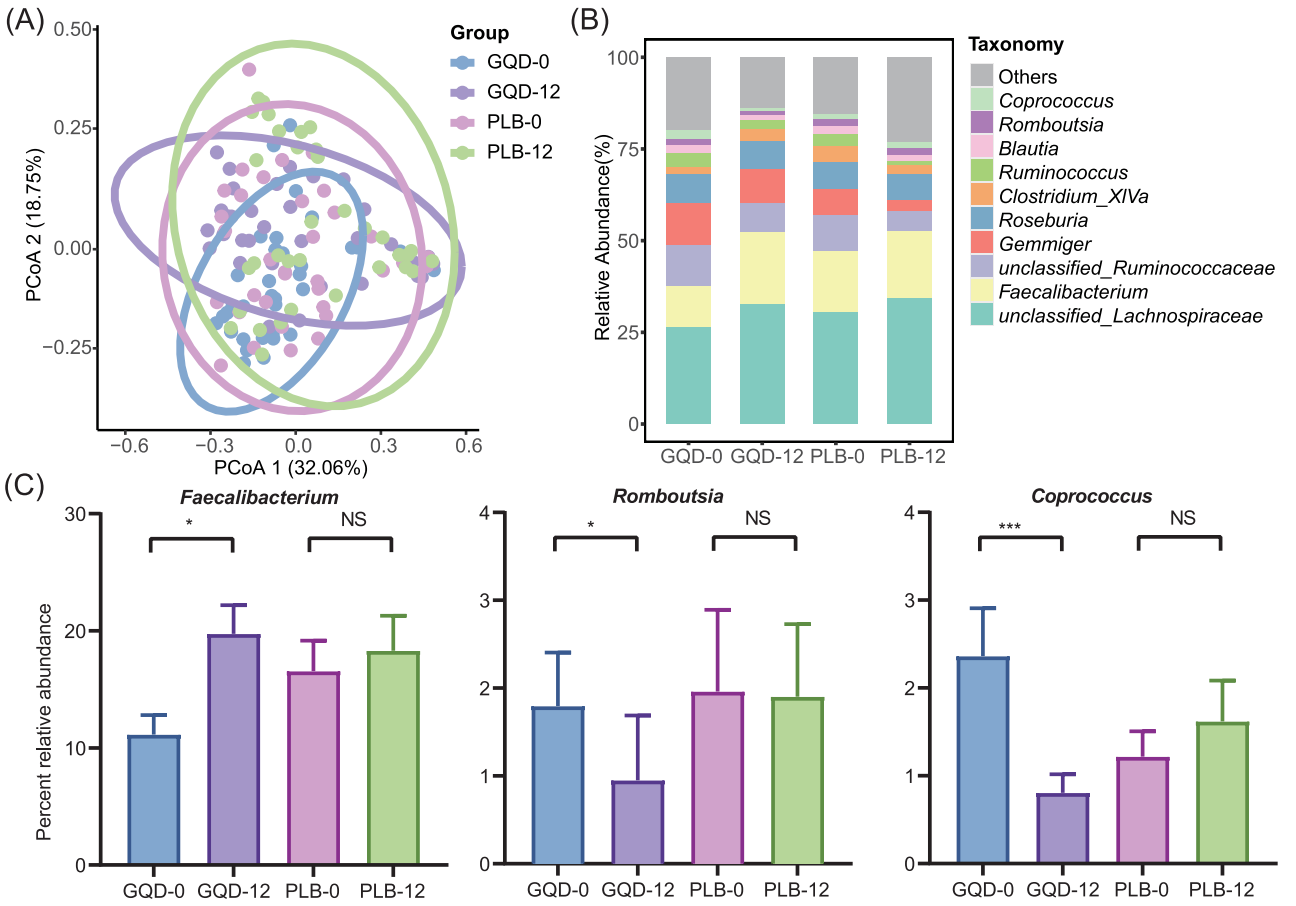


Figure 4. Changes in fecal microbial compositions after GQD intervention. (**A**) PCoA of the fecal microbiota based on Bray–Cutis distance. (**B**) Relative abundances of the 10 most abundant genera. "Unclassified" denotes an unclassified higher taxonomic level. (**C**) Effects of GQD or PLB treatment on the relative abundance of *Faecalibacterium*, *Romboutsia*, and *Coprococcus* in feces obtained at 12 weeks. $^{*}P < 0.05$, $^{***}P < 0.001$, NS: no significant difference (Wilcoxon signed-rank test). GQD-0/12: GQD sample at baseline (0 weeks)/12 weeks. PCoA: Principal coordinate analysis; PLB-0/12: PLB sample at baseline (0 weeks)/12 weeks.

clinical phenotypes, including HbA1c, FPG, CHO, and tryglyceride, which are key treatment parameters (Fig. 6, and Fig. S6A–D, see online supplementary material). Furthermore, GIP, which stimulates insulin secretion and is a potential target for treating T2DM [14], positively correlated with *Faecalibacterium* abundance (Fig. S6I). *Faecalibacterium* abundance also positively correlated with serum SCFAs, including acetic acid, butyric acid, and isocapric acid (Fig. S6E–H), and negatively correlated with serum endotoxin and pro-inflammatory cytokines, such as IL-1β and GM-CSF (Fig. S6J–L). Negative correlations between *Faecalibacterium* and 2h-PBG, LDL-C, endotoxin, TNF-α, IFN-γ, IL-6, IL-10, IL-17A, and MCP-1 were also identified, as well as positive correlations between *Faecalibacterium* and isobutyric acid, methylbutyric acid, valeric acid, and isocapric acid (Fig. S7, see online supplementary material). In conclusion, *Faecalibacterium* is central in the network of clinical phenotypes, metabolites, cytokines, and gut microbiome markers. Therefore, we hypothesized that *Faecalibacterium* is the central mediator of the hypoglycemic and anti-inflammatory effects of GQD.

Faecalibacterium recapitulates diabetes-alleviating effects in a T2DM mouse model

We used a mouse model to examine how *Faecalibacterium* alleviates metabolic disorders, which we established as a key gut microbial species enriched by GQD in humans. We chose *F. prausnitzii* (DSMZ 17677) as a representative species, which we administered for 10 weeks (5×10^{11} cfu/kg) to T2DM to explore its effects.

The weight of the obese T2DM mice at each time point was significantly higher than those of the control mice ($P < 0.01$, one-way ANOVA; Fig. 7A). However, the weight and epididymal adipose mass were significantly lower in the *F. prausnitzii*-treated diabetic mouse group than in the untreated diabetic group after 10 weeks ($P < 0.01$, one-way ANOVA; Fig. 7A, B). Serum CHO, LDL-C, and FPG levels were also lower in the *F. prausnitzii*-treated group than in the untreated group (Fig. 7C, D, F). The oral glucose tolerance test (Fig. 7G) and its areas under the blood glucose curve (Fig. 7E) were also better in the treated group than in the untreated group ($P < 0.01$, one-way ANOVA test). Overall, these results imply that *F. prausnitzii* supplementation recapitulates the effects of GQD treatment, including reducing body weight and alleviating insulin resistance.

Discussion

Diabetes is closely correlated with changes in the composition and function of the gut microbiota [9]. This study demonstrated that GQD treatment positively shifts the composition and function of the gut microbiome via a double-blinded RCT in patients with incipient diabetes and an animal study. Specifically, through combining the clinical efficacy evaluation of RCT and further

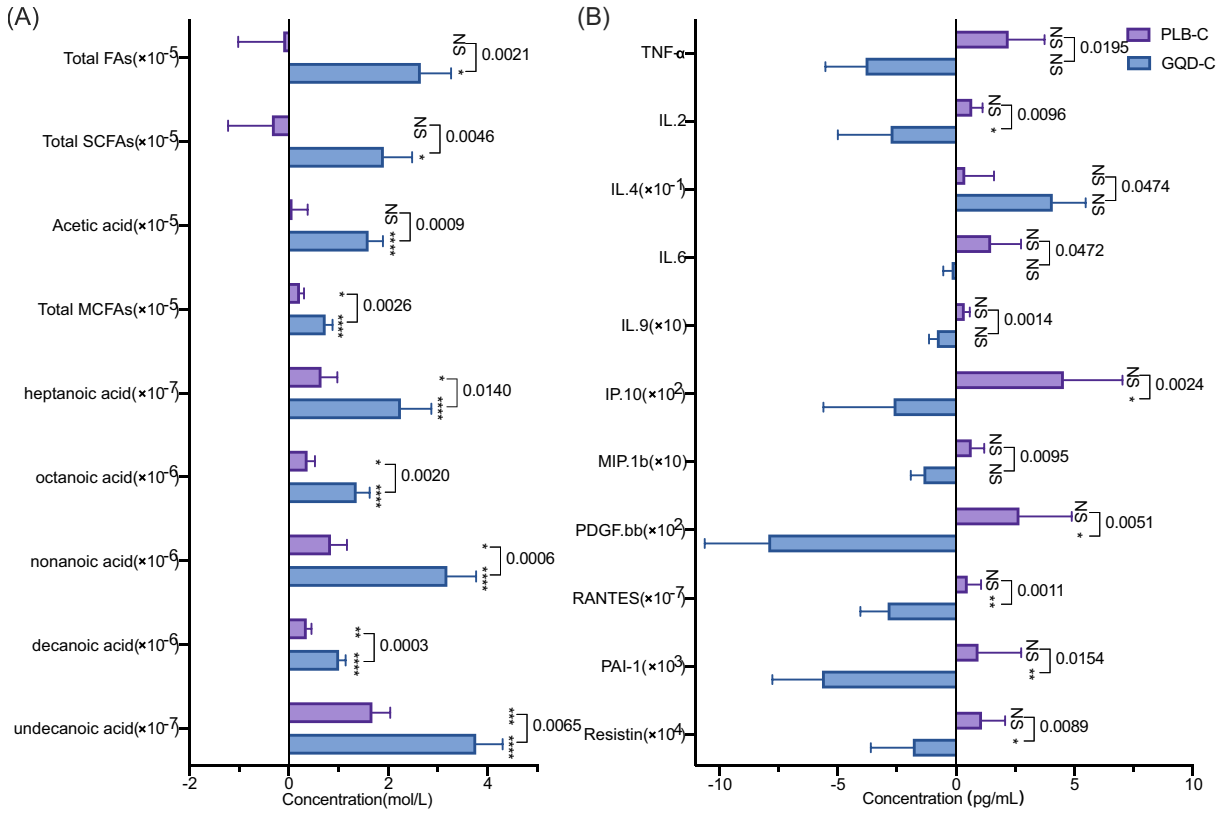


Figure 5. Effects of GQD intervention on serum metabolites and inflammation indicators. (**A**) Effects of GQD intervention on serum SCFA and MCFA levels. Serum acetic acid (an SCFA) and heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, and undecanoic acid (MCFAs) significantly changed after treatment. (**B**) Changes in serum cytokine and diabetes-associated biomarker levels from baseline (week 0) to week 12. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (unpaired or paired t-tests, Mann–Whitney U test, or Wilcoxon signed-rank test, as appropriate). GQD-C: GQD group change; FA: fatty acid; NS: not significant; PLB-C: PLB group change.

validation of Bayesian analysis, the RCT demonstrated that GQD significantly reduces HbA1c, FPG, 2h-PBG, HOMA-IR, weight, BMI, and LDL-C levels. It indicated the comprehensive effect of anti-diabetes and anti-obesity. The subsequent integrated multi-omics analyses identified *Faecalibacterium* as the key gut bacteria modulated by GQD. A correlational analysis indicated that the central role of *Faecalibacterium* correlated with phenotypic, metabolic, and immunological improvements in patients with T2DM, and these findings were validated in the diabetic mouse model.

This study found altered microbial compositions in the fecal samples of patients with diabetes treated with GQD, indicating that GQD influenced the gut microbiota, which mirrors previous studies in Goto–Kakizaki rats [10]. At the genus level, the relative abundances of *Romboutsia*, *Coprococcus*, and *Faecalibacterium* significantly changed after GQD intervention. *Romboutsia* is a member of the *Peptostreptococcaceae* family and is enriched in inflammatory bowel disease and Alzheimer's disease, which aggravate chronic inflammation [15,16]. Unlike previous reports [10], we found that the abundance of *Coprococcus*, a butyric acid-producing bacterium [17], differed after GQD treatment, but this discrepancy could be due to study population differences. Notably, we found the abundance of *Faecalibacterium* was significantly enriched after GQD intervention, similar to numerous previous studies [9,10]. Lower abundances of *Faecalibacterium* have been identified in diseases such as obesity, diabetes [18], metabolic liver disease [19], and inflammatory bowel disease [20]. As a butyrate producer, *Faecalibacterium* provides energy to intestinal epithelial cells, maintains tight junctions, regulates intestinal mucosal immunity, and alleviates insulin resistance [21,22]. The correlation network analysis in this study determined that the abundance of GQD-enriched *Faecalibacterium* positively correlated with serum butyric acid levels, replicating previous findings [10]. Interestingly, acetic acid, another SCFA, was positively associated with *Faecalibacterium* richness in this study, and the serum levels of acetic acid significantly increased after GQD intervention. Acetic acid improves metabolic diseases, such as diabetes, primarily by regulating liver lipid metabolism and insulin sensitivity through free fatty acid receptor 2 and alleviating local inflammation of islets to promote insulin secretion. Furthermore, the increase in total SCFAs may help improve T2DM by activating G-coupled receptor 43 (GPR43), which inhibits insulin signaling in adipocytes and reduces fat accumulation [23].

This study also found a significant increase in MCFAs after GQD treatment, which are fatty acids with 6–12 carbons mainly derived from the diet. MCFAs regulate glucose and lipid metabolism through multiple pathways *in vivo*. For instance, they activate GPR40 in pancreatic beta cells and promote insulin secretion through the inositol triphosphate/Recombinant Stromal Interaction Molecule 1/Calcium release-activated calcium channel protein 1 pathway to modulate glucose metabolism. They also affect insulin sensitization, glucose-stimulated insulin secretion, and gluconeogenesis by activating peroxisome proliferator-activated receptor gamma [24]. MCFAs also mitigate lipid accumulation in liver, adipose tissue, and skeletal muscle cells [25]. Therefore, our findings provide additional mechanistic insights into

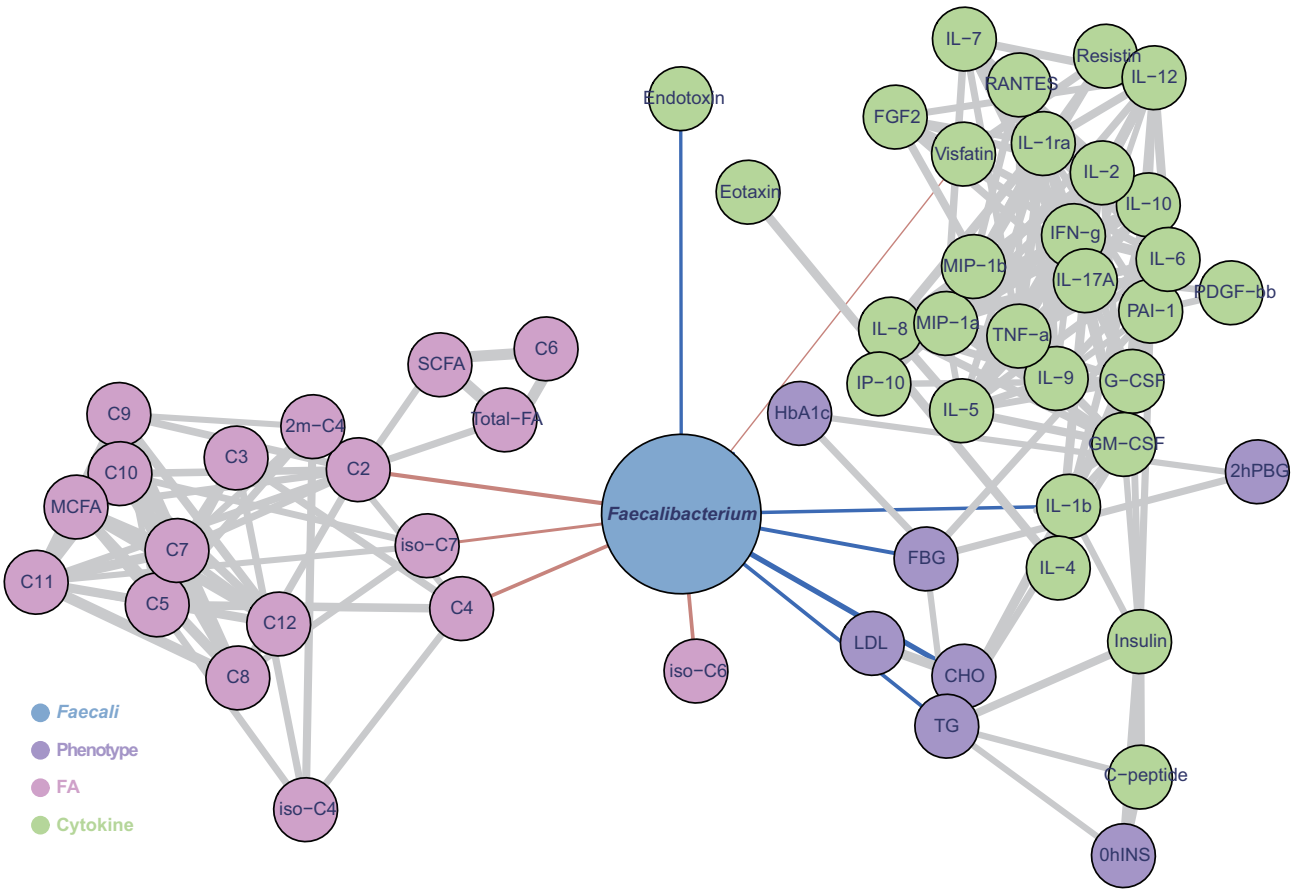


Figure 6. Network plot showing the correlational network centered on *Faecalibacterium* and host phenotypes, including clinical traits and serum metabolites. Correlations with q-values < 0.1 are shown. The Spearman's correlation coefficient (R) is indicated by the thickness of the lines. Red lines indicate positive associations with *Faecalibacterium* and blue lines indicate negative associations. Light grey lines indicate strong associations between factors other than *Faecalibacterium* (the absolute value of $R > 0.5$). 2m-, 2-methyl-; C2, acetic acid; C3, propionic acid; C4, butyric acid; C5, valeric acid; C6, capric acid; C7, heptanoic acid; C8, octanoic acid; C9, nonanoic acid; C10, decanoic acid; C11, undecanoic acid; C12, lauric acid.

the modulation of the gut microbiome and host metabolism by GQD.

Moreover, GQD treatment alleviated systemic inflammation in patients with T2DM, evidenced by decreased cytokine levels. TNF-α, IL-6, IP-10, MIP-1b, and RANTES are related to macrophage activity. Specifically, MIP-1b, RANTES, and IP-10 (also referred to as CCL4, CCL5, and CXCL10, respectively) function as chemokines, facilitating the recruitment and pro-inflammatory phenotypes of macrophages, as well as inducing the secretion of TNF-α and IL-6 [26]. TNF-α induces chronic low-level inflammation and causes infiltration of inflammatory cells into the pancreas, liver, and adipose tissue, aggravating insulin resistance and damaging insulin secretion. IL-2 and IL-9 are pro-inflammatory cytokines associated with T2DB [27]. We also found reduced PAI-1 and resistin levels, further supporting that GQD treatment alleviated vascular endothelial dysfunction in patients with T2DM [28] and improved insulin resistance by inhibiting resistin secretion [29].

The complex "gut microbiota–metabolism–inflammation–diabetes" axis is an important direction for understanding the mechanisms of diabetes and discovering novel intervention targets [30]. An altered gut microbial composition affects the generation of SCFAs, secondary bile acids, aromatic amino acids, branched-chain amino acids, and other metabolites [5]. These metabolites, in turn, are important for regulating the body's systemic immunity and metabolism. In this study, *Faecalibacterium* enrichment after GQD intervention correlated with increasing serum concentration of total SCFAs and negatively correlated with pro-inflammatory cytokines, such as IL-1β, GM-CSF, and endotoxin.

More and more studies have found that the abundance of *F. prausnitzii*, as the core strain of *Faecalibacterium*, is significantly decreased in both T1DM and T2DM patients [31]. A systematic review of observational studies, in which a total of 18 studies (5489 participants) were included, showed a decreased abundance of *F. prausnitzii* in prediabetes and newly diagnosed T2DM [32]. Besides, when GLP-1 receptor agonists such as benaglutide and fecal microbiota transplantation of healthy participants are used to treat T2DM, it has been found that *F. prausnitzii* shows a significant enrichment trend [33–35]. This is consistent with our results after GQD intervention. Therefore, *F. prausnitzii* is intrinsically correlated with insulin resistance and glucose metabolism disorders in T2DM [36]. Effectively restoring the relative abundance of *F. prausnitzii* is beneficial in alleviating T2DM. However, at present, there is no research evidence on the intervention of *F prausnitzii* in T2DM treatment. To verify this hypothesis, we administered *F. prausnitzii* to mice with T2DM, finding that it recapitulated the effects of GQD, especially by reducing HbA1c and FPG levels and lipid levels, such as CHO and triglyceride. Thus, this not only indicated that *F. prausnitzii* contributes to the mitigation of T2DM during GQD intervention, but also provides evidence that *F. prausnitzii* may also be a potential anti-diabetic probiotic.

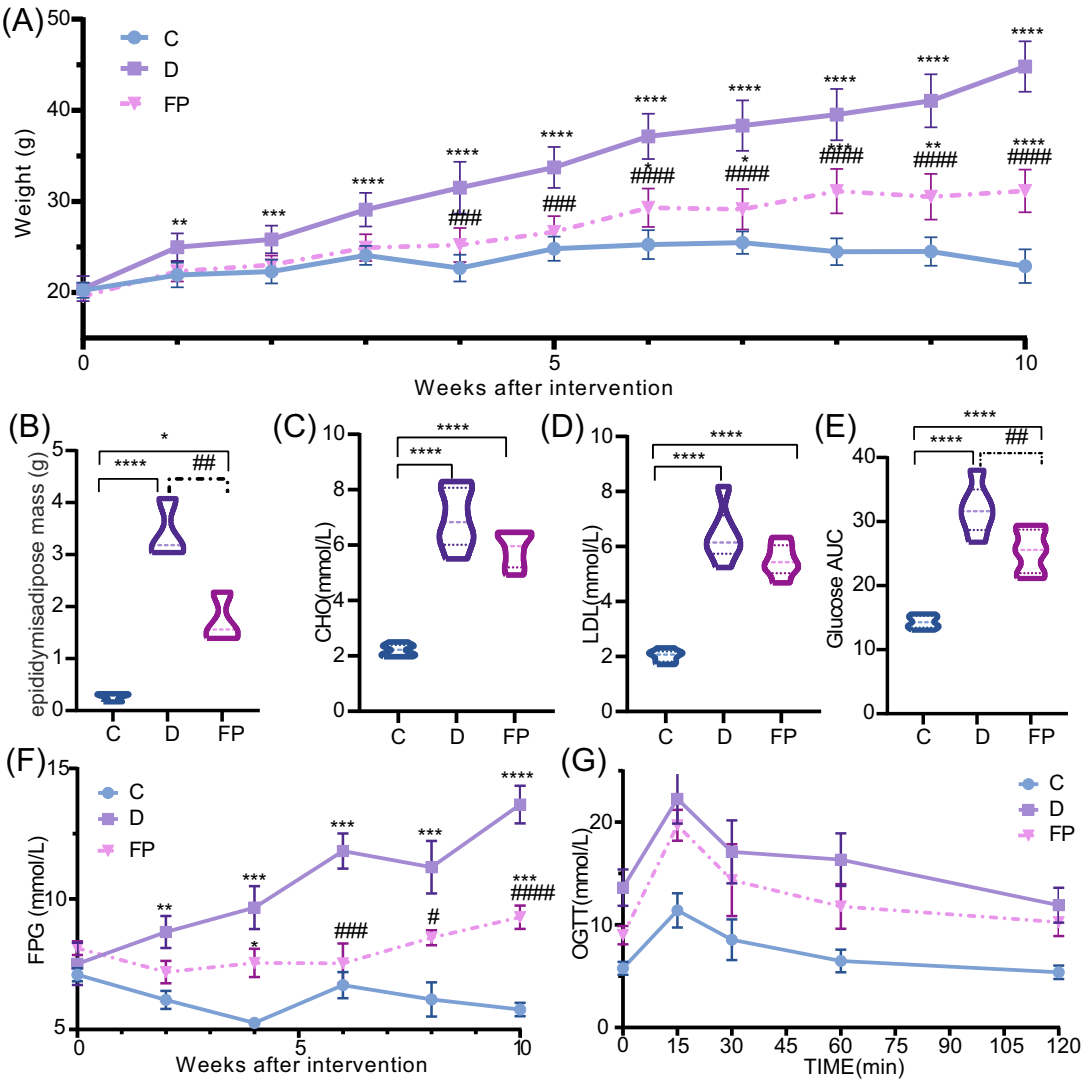


Figure 7. Effects of *Faecalibacterium* supplementation for 10 weeks in obese T2DM mice. (**A**) Body weight changes over time. (**B**) Epididymal fat changes. (**C**) CHO level changes. (**D**) LDL-C level changes. (**E**) Areas under the blood glucose curve during OGTTs. (**F**) FPG level changes over time. (**G**) OGTT changes after oral glucose administration (2 g/kg). *Significantly differs from the C group, #significantly differs from the D group (*,#$P < 0.05$, **,##$P < 0.01$, ***,###$P < 0.001$, ****,####$P < 0.0001$; one-way ANOVA with Tukey's multiple comparison test). C, Control group (non-diabetic mice); D, diabetic group; FP, diabetic mice treated with *F. prausnitzii*; OGTT, oral glucose tolerance test.

Conclusions

This study demonstrated that GQD treatment alleviates T2DM by improving metabolic disorders and systemic inflammation through gut microbiome modulations. *Faecalibacterium* and SCFAs were central to the metabolic and immunological improvements in GQD-treated patients and mice. Our future studies will continue to investigate how complex traditional Chinese medicine formulas affect T2DM and other metabolic disorders.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grants No. 81430097, 81973837, 82004242, 82274343 and 31771481) and the Innovation Team and Talents Cultivation Program of National Administration of Traditional Chinese Medicine (Grant No. ZYYCXTD-D-202001). The authors wish to thank Dr. Feng Tian for the advice on clinical data analysis.

Author contributions

L.H.Z., X.L.T., and J.W. co-supervised the study. L.H.Z., X.L.T., L.S.H., and H.W. co-designed the RCT trials; L.S.H, Z.Z.G., H.W., Y.W.L., C.J.G., M.Y.W., and X.H.H. co-completed the RCT trials and sample collection; Y.F.L. completed the Bayesian analysis; Z.Z.G., L.S.H., H.W., and S.D. co-completed the collation of clinical data and multi-omics detection; X.L.T., L.H.Z., X.T.J., X.M.W., and L.H. co-designed and completed the animal experiment of microbial transplantation; L.C. isolated and cultured *Faecalibacterium prausnitzii*. W.H.Z., Z.Z.G., and X.Z. analyzed the data and prepared the figures. X.L.T., L.H.Z., and J.W. co-designed the manuscript based on the clinical and sequencing data. Z.Z.G., W.H.Z., and L.S.H. wrote and revised the manuscript and all authors proofread and improved the manuscript.

Supplementary data

Supplementary data is available at *PCMEDI Journal* online.

Conflicts of interest

All authors declare that they have no conflict of interest or financial conflicts to disclose. In addition, as an Editorial Board Member of *Precision Clinical Medicine*, the corresponding author Jun Wang was blinded from reviewing and making decision on this manuscript.

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Received 7 December 2023; accepted 6 February 2024. published 14 February 2024