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Impact of Lactobacillus johnsonii on glycemic control and lipid metabolism in Type 2 Diabetes with Circadian Disruption

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Abstract

Objective: Although most patients with type 2 diabetes mellitus (T2DM) and circadian rhythm disruption have poor blood glucose control, a fraction of patients with T2DM and circadian rhythm disruption who still have good blood glucose control. Previous studies have shown that individuals with circadian rhythm disruption are more prone to developing T2DM, and the occurrence of T2DM is associated with the gut microbiota. However, the role of gut microbiota in patients with T2DM and circadian rhythm disruption remains unclear.

Methods: Stool samples were collected from 6 patients with poorly controlled type 2 diabetes mellitus (T2DM) and circadian rhythm disruption, as well as from 6 patients with well-controlled T2DM and circadian rhythm disruption. Metagenomic sequencing was performed on the stool samples. Compared to the well-controlled group, the abundance of *Lactobacillus johnsonii(L. johnsonii*) was significantly decreased in the poorly controlled group. To investigate the effects of L. johnsonii supplementation on glucose and lipid metabolism, diabetic mice with circadian rhythm disruption were administered L. johnsonii and their metabolic indicators were measured.

Results: Metagenomic sequencing of the gut microbiota revealed a higher microbial diversity in the well blood glucose controlled type 2 diabetes combined with disrupted circadian rhythm group (W-T2D-RD). Additionally, a significant decrease in the abundance of *L. johnsonii* was observed in patients with poor blood glucose controlled type 2 diabetes combined with disrupted circadian rhythm group (P-T2D-RD) when compared to those with W-T2D-RD. Following supplementation of *L. johnsonii* to the mice in the type 2 diabetes mellitus rhythm disruption *Lactobacillus johnsonii* group (T2DM-RD-L), the fasting blood glucose levels and postprandial blood glucose levels were

significantly reduced. Additionally, total cholesterol and low-density lipoprotein levels decreased, high-density lipoprotein levels increased in the T2DM-RD-L group.

Conclusion: *Lactobacillus johnsonii* has a positive impact on both glucose and lipid metabolism in patients with type 2 diabetes mellitus and circadian rhythm disruption.

Keywords: *Lactobacillus johnsonii*; metagenomics; type 2 diabetes mellitus; blood glucose control; circadian rhythm disruption

1. Introduction

Type 2 diabetes mellitus (T2DM) is the most common metabolic disorder characterized by high blood glucose, hyperlipidemia, and insulin resistance [1]. According to the latest statistics from the International Diabetes Federation (IDF), the global prevalence of diabetes among individuals aged 20-79 was approximately 536.6 million in 2021, projected to rise to 783.2 million by 2045[2]. Among them, China has the highest number of diabetes patients within the 20-79 age group worldwide. Diabetes can lead to various serious complications[3], such as coronary artery disease, peripheral arterial disease, retinopathy, and diabetic nephropathy, imposing a heavy economic burden on families and society.

The recognized risk factors for diabetes include obesity, unhealthy lifestyle habits (such as irregular diet and sleep patterns), family history, and genetics [4]. With the development of social modernization and industrialization, shift work has become increasingly common, with approximately one-fifth of workers globally engaged in night shifts [5]. Occupations such as police, firefighting, healthcare, transportation, communications, energy, and water supply require rotating shifts due to the need for continuous 24-hour operation. Large-scale epidemiological studies have shown that shift work is an independent risk factor for type 2 diabetes [6-8]. Research has demonstrated that desynchronization of the circadian rhythm system with the external environment, also known as "circadian rhythm disruption," is detrimental to human health, and the misalignment between the central circadian "clock" and daily behavior is associated with an increased incidence of T2DM [9, 10].

The gut microbiota is considered a complex ecosystem in the gastrointestinal tract, composed of bacteria, archaea, fungi, viruses, and protozoa [11, 12]. In recent years, increasing evidence has shown a significant association between the gut microbiota and type 2 diabetes [13-17]. Studies have also demonstrated a link between circadian rhythm disruption and poor blood glucose control in patients with type 2 diabetes [18-20]. Furthermore, current research suggests that circadian rhythm disruption, such as shift work, is associated with changes in the gut microbiota [21-23]. The gut microbiota plays an important role in T2DM, participating in the regulation of glucose metabolism, influencing insulin sensitivity, and improving symptoms in diabetic patients[13-15, 24, 25]. Fecal microbiota transplantation (FMT) is an effective and safe method

that can modulate the composition of the gut microbiota, thereby alleviating high blood glucose levels in patients. However, there are currently no studies investigating the relationship between blood glucose control and gut microbiota in patients with type 2 diabetes complicated by circadian rhythm disruption. Therefore, we aim to collect fecal samples from individuals with type 2 diabetes and circadian rhythm disruption for metagenomic sequencing to provide evidence for the use of gut microbiota transplantation in the treatment of poorly controlled blood glucose in these patients.

2. Materials and methods

2.1 Study Participants

Inclusion Criteria:

(1) Diagnosis of type 2 diabetes according to the 1999 WHO criteria: fasting blood glucose (FBG) ≥ 7.0 mmol/L or 2-hour postprandial blood glucose (PBG) ≥ 11.1 mmol/L;

(2) Age between 30 and 55 years;

(3) Participants with circadian rhythm disruption, primarily those engaged in shift work[26]. Shift work was defined as work outside of normal working hours (7:00-18:00), or rotating shifts occurring between 7:00 PM and 6:00 AM. Participants who have not worked in shifts or night shifts for the past 6 months were considered non-shift workers. Night shift work frequency was categorized as 1-2, 3-4, or ≥ 5 night shifts per month, while night shift work duration was categorized as <10, 10-19, or ≥ 20 years of night shift work;

(4) Informed consent obtained as required by the research ethics committee.

Exclusion Criteria:

(1) Type 1 diabetes (T1DM) or secondary diabetes caused by pancreatic damage or other conditions such as Cushing's syndrome, thyroid abnormalities, or acromegaly. Participants with acute complications of diabetes or severe chronic complications were also excluded;

(2) Use of antibiotics or other probiotics or probiotic products within the past 3 months;

(3) Severe organic heart disease, including but not limited to congenital heart disease, rheumatic heart disease, hypertrophic or dilated cardiomyopathy;

(4) Recent use of corticosteroids orally, intramuscularly, intravenously, or non-gastrointestinal joint injection within the past 3 months;

(5) History of malignancy within the past 5 years, regardless of evidence of recurrence or metastasis;

(6) Severe hepatic dysfunction, defined as serum alanine aminotransferase concentrations exceeding 2.5 times the upper limit of the normal range;

(7) Impaired renal function, defined as serum creatinine > 132μmol/L-1 or estimated glomerular filtration rate (eGFR) < 60 mL;

(8) Digestive system diseases such as Crohn's disease, ulcerative colitis, irritable bowel syndrome, or symptoms of malabsorption, chronic diarrhea, or

severe constipation;

(9) History of intestinal resection or other gastrointestinal surgery (e.g., cholecystectomy) within the past year, or non-gastrointestinal surgery within the past 6 months;

(10) Mental illness, severe infection, severe anemia, or neutropenia;

(11) Participation in other clinical trials within the past 3 months, poor compliance, or inability to complete follow-up;

(12) Pregnant women.

2.2 Grouping Scheme

Patients were collected through questionnaires, and then randomly numbered and enrolled. After obtaining informed consent from each volunteer, we conducted fasting blood glucose (FPG), glycated hemoglobin (HbA1C), triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) tests on the recruited volunteers. These tests were conducted by the laboratory department of our hospital.

Participants with FBG > 7.0 mmol/L or PBG > 11.1 mmol/L and HbA1c > 7.0% were categorized into the poorly controlled type 2 diabetes combined with disrupted circadian rhythm group (P-T2D-RD).

Participants with FBG < 7.0 mmol/L or PBG < 11.1 mmol/L and HbA1c < 7.0% were categorized into the well-controlled type 2 diabetes combined with disrupted circadian rhythm group (W-T2D-RD).

These studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice and were approved by the Ethics Committee of Hebei University of Engineering affiliated hospital (2022 □ K □ 058).All participants provided informed consent by signing the consent form.

2.3 Collection of fecal samples for metagenomic sequencing.

We collected fasting stool samples of approximately 2-5g. The samples were then immediately placed in a cold storage box to maintain a low temperature and transported to the laboratory as soon as possible. Upon arrival at the laboratory, the samples were transferred into 15ml sterile centrifuge tubes, with each tube containing 1-2g of stool. The tubes were tightly sealed with caps and the opening was covered with sealing film to maintain an anaerobic environment as much as possible. The stool samples were rapidly frozen in liquid nitrogen for 5 minutes and subsequently stored at -80℃ in an ultra-low temperature freezer to prevent repeated freeze-thaw cycles. The fecal samples were used for microbiome analysis using metagenomic sequencing, which was conducted by Novogene.

The purity and integrity of the DNA were analyzed using 1% agarose gel electrophoresis (AGE). DNA quantification was performed using the Qubit® dsDNA Assay Kit with the Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). For library construction, 1ug of genomic DNA was taken and processed using the NEBNext® Ultra DNA Library Prep Kit for Illumina (NEB, USA). The DNA was randomly fragmented to approximately 350 bp in length using a Covaris sonicator, followed by end repair, A-tailing, adapter ligation, purification, and

PCR amplification to complete the entire library preparation process. After quality assessment of the libraries, different libraries were pooled based on their effective concentrations and the required data output for sequencing, and then subjected to Illumina PE150 sequencing.

2.4 Animal experiments.

Thirty SPF-grade healthy male Kunming mice (Beijing Weitonglihua Experimental Animal Technology Co., Ltd.) were used in this study. The mice were 3 weeks old with a body weight range of 18-20g. They were housed in the animal facility of the Center Laboratory at Hebei University of Engineering affiliated hospital. After a one-week adaptation period, the mice were randomly divided into the following five groups, with 6 mice in each group. For the diabetic mice: Group 1 served as the type 2 diabetes mellitus control group (T2DM-C); Group 2 served as the type 2 diabetes mellitus with rhythm disruption group (T2DM-RD); Group 3 served as the type 2 diabetes mellitus with rhythm disruption and *L. johnsonii* intervention group (T2DM-RD-L). For the normal mice: Group 4 served as the normal control group (C); Group 5 served as the normal mice with disrupted sleep-wake rhythm group (CRD).

After 5 weeks of high-fat diet feeding, the mice in the diabetes groups were fasted but allowed access to water for 18 hours. They were then intraperitoneally injected with streptozotocin (STZ, 100mg/kg) dissolved in 0.05 M sodium citrate buffer (pH 4.5). Three days after the injection, tail blood samples were obtained from the mice for blood glucose measurement using a glucometer. Random blood glucose levels ≥16.7mmol/L indicated successful modeling of diabetes. After successful modeling, the mice in the CRD, T2DM-RD, and T2DM-RD-L groups were subjected to a combination of light-dark cycle changes and sleep deprivation using a mouse sleep deprivation apparatus to disrupt their normal circadian rhythm. The light-dark cycle was as follows: dark phase (8:00-20:00) and light phase (20:00-8:00). This cycle was repeated for 3 consecutive days, and on the 4th day, the light-dark cycle was reversed, with the dark phase from 8:00-20:00 and the light phase from 20:00-8:00. This cycle was also repeated for 3 consecutive days, lasting a total of 6 days, which served as one complete cycle. The sleep deprivation intervention was conducted daily from 8:00 to 20:00 using the mouse sleep deprivation apparatus. On the 13th day of the circadian intervention, the mice in the T2DM-RD-L group were orally gavaged with *L. johnsonii* for 42 days, while the other groups received oral gavage of saline solution. All experimental protocols were approved by the Hebei University of Engineering affiliated hospital Institutional Animal Care and Use Committee (IACUC). Research was conducted in accordance with all relevant guidelines and regulations of the Hebei University of Engineering affiliated hospital , IACUC(IACUC-Hebeu-2023-0006) and all methods are reported in accordance with ARRIVE guidelines.

2.5 Cultivation of *Lactobacillus johnsonii*.

To prepare the *L. johnsonii* (provided by BeNa Culture Collection), the freeze-dried vial was opened in an anaerobic incubator. The surface of the vial

was disinfected by gently wiping it with a 75% alcohol swab. The vial was then crushed using forceps, and 0.5mL of liquid culture medium was drawn into the vial, ensuring complete dissolution. The solution was transferred back into a liquid centrifuge tube, mixed well, and 0.2mL of bacterial suspension was pipetted onto a Petri dish and spread evenly. This process was repeated to obtain two Petri dishes. All the liquid tubes and Petri dishes were placed in an anaerobic incubator at 37℃ for 48 hours until the colonies grew. Afterward, a 1% inoculum was prepared by transferring the colonies to a liquid culture medium and incubating them at 37℃ for 48 hours. This process was repeated for two generations to activate the strain. Finally, using the colony counting method, the total viable count was adjusted to 2.4×10^9 CFU/mL.

2.6 Detection indicators.

Glucose metabolism indicators:

During the experiment, random blood glucose levels of each group of mice are measured every 6 days, and fasting blood glucose levels of the mice are recorded once a month. Tail blood samples are collected, and a rapid blood glucose meter and blood glucose test strips are used for testing. Insulin (INS) levels are measured using an ELISA assay kit.

Lipid metabolism indicators:

TG, TC, LDL, and HDL are measured using commercial assay kits, following the instructions provided.Collect mice blood samples through cardiac puncture after administering anesthesia via inhalation,then this mice were euthanized with CO_2 gas(30%,2L/min).

2.7 Statistical analysis

The statistical analysis figures were generated using Origin 8.1 software. Data collection and entry were performed using Excel software, while statistical analysis was conducted using IBM SPSS 25.0 statistical software. Experimental data for continuous variables were expressed as means ± SD, while categorical data were presented as frequencies or rates.The differences in laboratory testing between two groups were compared using independent sample t-tests. Chi-squared test were used for comparisons of categorical among groups. For the comparison of continuous variables among multiple groups, analysis of variance (ANOVA) was employed. In case of homogeneity of variance, one-way ANOVA followed by the least significant difference (LSD) test was used for intergroup comparisons. In the case of heterogeneity of variance, Tamhane's T2 test was used for pairwise comparisons. A difference was considered statistically significant when the p values□0.05.

3 Results

3.1 Participant Characteristics

According to the inclusion and exclusion criteria, a total of 12 patients with T2DM and circadian rhythm disorders were recruited from the Endocrinology Department of Hebei Engineering University Affiliated Hospital between July 2022 and May 2023. Among them, 6 patients were included in P-T2D-RD group, and 6 patients were included in W-T2D-RD group. General clinical data are

presented in Table 1. All recruited patients were males, and there were no significant differences in height and disease duration between the two groups. The body weight and body mass index (BMI) in the P-T2D-RD group were higher than those in the W-T2D-RD group, but the differences were not statistically significant ($p>0.05$). Fasting blood glucose, glycated hemoglobin, cholesterol, and low-density lipoprotein levels in the P-T2D-RD group were significantly higher than those in the W-T2D-RD group, and the differences were statistically significant ($p<0.05$). There was no significant difference in triglycerides and high-density lipoprotein levels between the two groups.

Table 1 Clinical characteristircs of the participants

Characteristics	W-T2D-RD	P-T2D-RD	p
Number	6	6	-
Male/female	6/0	6/0	-
Age (year)	43.67±5.99	39±5.44	0.188
Height (cm)	1.74±0.03	1.76±0.05	0.56
BMI (kg/m²)	24.69±4.09	27.50±2.31	0.18
Duration (years)	5.42±5.21	6±3.40	0.82
HbA1C (%)	6.27±0.67	7.82±0.58□□	0.002
FPG (mmol/L)	6.53±1.40	8.34±1.30□	0.043
TC (mmol/L)	3.84±0.85	5.15±0.72□	0.017
TG (mmol/L)	1.17±0.77	2.74±1.76	0.087
HDL (mmol/L)	1.16±0.17	1.19±0.16	0.710
LDL (mmol/L)	2.24±0.77	3.12±0.55	0.049
No drinking/drinking	4/2	3/3	0.56
No smoking/smoking	3/3	4/2	0.56
hypoglycemic drugs (a/b)	1/5	4/2	0.08
No eating on time/ eating on time	1/5	4/2	0.08
No exercise /exercise	1/5	3/3	0.22

□*P*□0.05□□□*P*□0.01□

Note:hypoglycemic drugs(a/b):insulin+oral hypoglycemic agents/oral hypoglycemic agents;poorly controlled type 2 diabetes combined with disrupted circadian rhythm group (P-T2D-RD),well-controlled type 2 diabetes combined with disrupted circadian rhythm group (W-T2D-RD).

3.2 Gut Microbiome Results

3.2.1 Overview of Metagenomic Results

A total of 12 fecal samples were collected, with 7 fecal samples sent for testing in December 2022 (including 3 poorly controlled blood glucose and 4 well-controlled blood glucose cases), and 5 fecal samples sent for testing in May

2023 (including 3 poorly controlled blood glucose and 2 well-controlled blood glucose cases). Due to the samples being sent for testing in different batches, a combined analysis of the results revealed no significant differences in species abundance based on the Anosim analysis. Therefore, the results from the first batch were selected for further metagenomic analysis.

3.2.2 Gene Prediction and Abundance Analysis

Based on the information obtained from the samples, statistical analysis was conducted on the gene features. Core gene analysis (Fig. 1A) and Pan gene analysis (Fig.1B) indicated that as the number of samples increased and the number of genes stabilized, the collected samples in this experiment were abundant and sufficient. To further analyze the reliability of the experiment and the rationality of sample selection, we conducted a correlation analysis of the samples (Fig. 1D). The results showed a high similarity in the gene abundance patterns among the samples, indicating good biological replicability of the experiment. The analysis of gene quantity differences revealed that W-T2D-RD group had more genes than P-T2D-RD group (Fig.1C), suggesting a richer microbial composition in the healthy control group compared to the T2DM patients. In addition, among all the genes in both groups, there were 407,876 shared genes between P-T2D-RD group and W-T2D-RD group, 58,462 unique genes in the P-T2D-RD group, and 270,483 unique genes in the W-T2D-RD group (Fig. 1E).

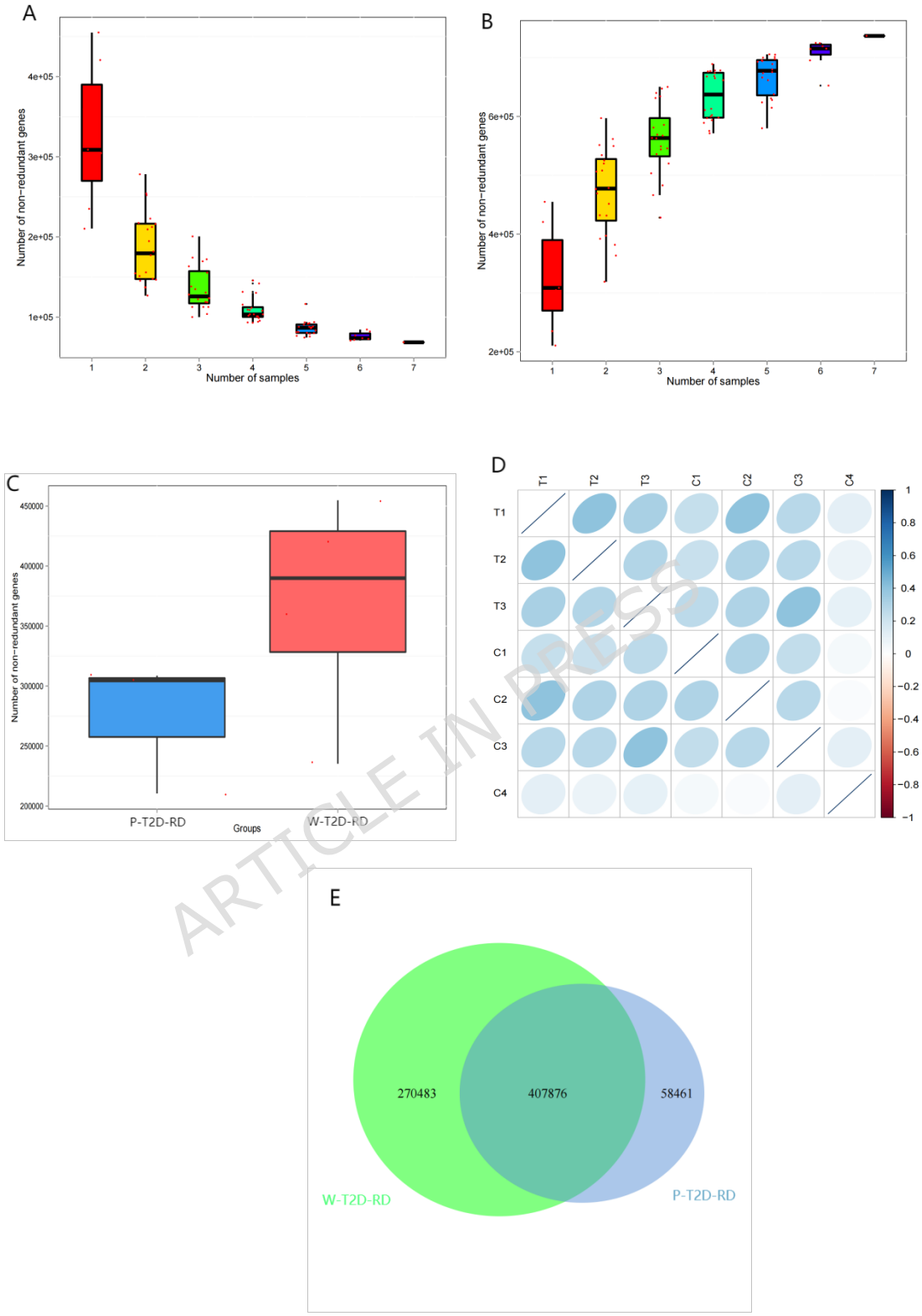


Figure.1 Gene prediction and abundance analysis of gut microbiota of two groups of people.A. Core gene rarefaction curve; B. Pan gene rarefaction curve; C. Boxplot of inter-group gene count differences; D. Heatmap of sample correlations; E. Venn diagram analysis of gene counts.

3.2.3 Comparison of Microbial Community Composition based on

Metagenomics

We explored the potential differences in gut microbiota between 3 cases of poorly controlled T2DM with circadian rhythm disorders and 4 cases of well-controlled T2DM with circadian rhythm disorders. At the phylum level, the dominant bacterial phyla in P-T2D-RD group included *Firmicutes (42.8%)*, *Actinobacteria (32.0%)*, *Bacteroidetes (16.2%)*, and *Proteobacteria (0.9%)*; while the dominant microbial phyla in W-T2D-RD group were *Firmicutes (62.9%)*, *Actinobacteria (11.9%)*, *Bacteroidetes (10.9%)*, and *Proteobacteria (4.7%)* (Fig. 2A). At the genus level, the dominant bacterial genera in P-T2D-RD group included *Bifidobacterium(29.2%), Bacteroides(10.2%),Faecalibacterium(7.5%)and Prevotella(3.2%)*; while the dominant bacterial genera in W-T2D-RD group included *Megamonas (11.4%), Bifidobacterium (8.9%), Clostridium (4.3%), Bacteroides (4.2%), Roseburia (3.4%)*, *Prevotella (3.2%and Faecalibacterium (3.1%)* (Fig. 2B). Sequences that could not be assigned to any known group and detected at low abundance were classified as "Other". This suggests that there are still a significant number of unknown bacteria in the human gut. Compared to the W-T2D-RD group, the abundance of *L.johnsonii* was significantly decreased in P-T2D-RD group (Figure. 2C).

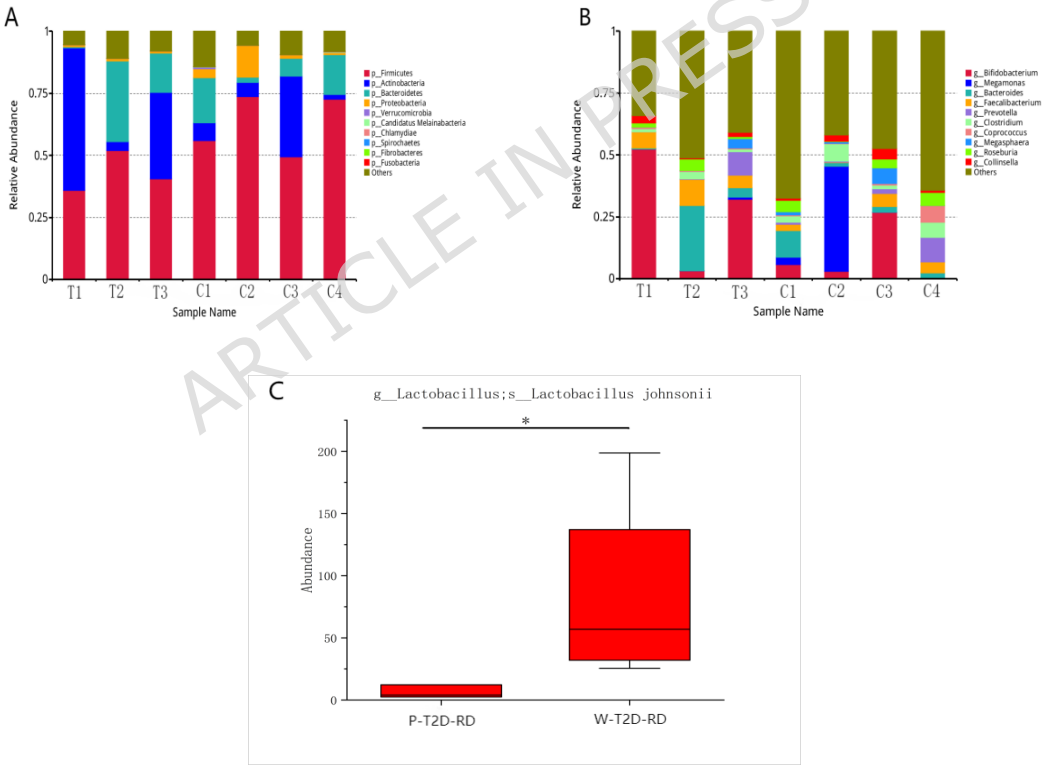


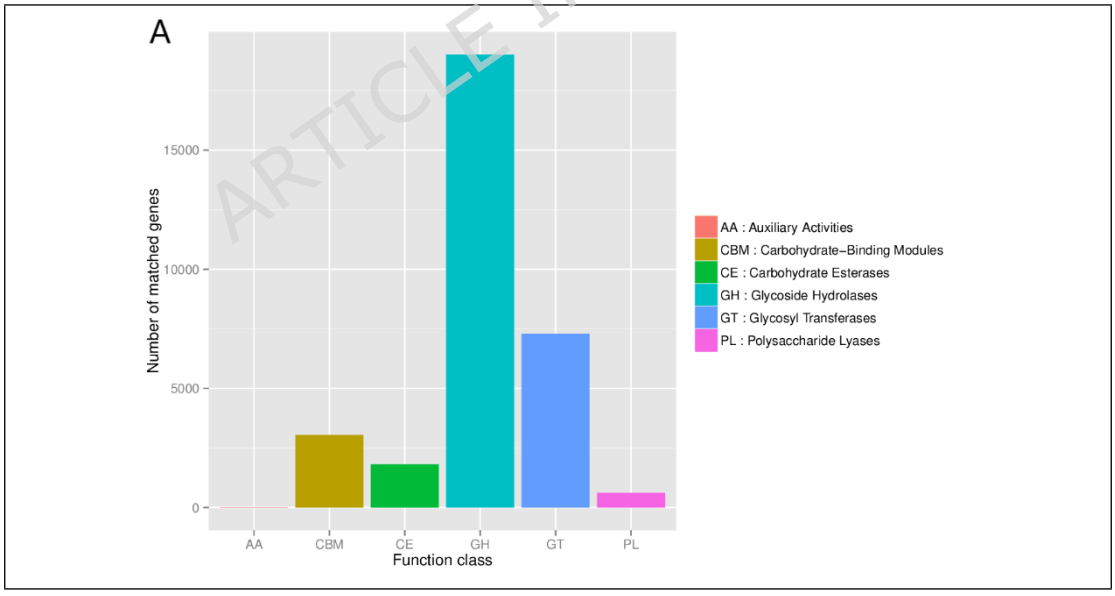
Figure.2 Comparison of microbial community composition in metagenomics.Note: A. Bar plot of relative abundance at the phylum level; B. Bar plot of relative abundance at the genus level; C. Boxplot presentation of significantly different species. ✱P□0.05.
P-T2D-RD:T1, T2, and T3; P-T2D-RD:C1, C2,C3, and C4.

3.2.4 Diversity profile of CAZymes

Carbohydrate-Active Enzymes (CAZymes) are a series of enzymes that

possess functions including degradation, modification, and synthesis of glycosidic bonds. Microorganisms can produce numerous CAZymes, working in conjunction with human cells to degrade a diverse array of complex carbohydrates, facilitating further metabolic utilization and the production of new bioactive signaling molecules. This process represents an important pathway through which microorganisms participate in the physiological and pathological processes of the human body.To determine the gut CAZymes profiles, we performed CAZymes analysis using the metagenomic data of the P-T2D-RD and the W-T2D-RD patients based on the CAZy database. In total, 31,783 putative genes were identified, and the majority of these genes were assigned to glycoside hydrolases (GH 19010, 59.8%), followed by glycosyl transferases (GT 7287, 22.9%), carbohydrate-binding modules (CBM 3049, 9.5%), carbohydrate esterases (CE 1811, 5.7%), polysaccharide lyases (PL 619, 1.9%), and auxiliary activities (AA 7, 0.02%) (Fig. 3A).

We identified 248 known CAZymes families, and they corresponded mainly to polysaccharide-degrading activities; these included 119 GHs, 49 GTs, 49 CBMs,16PLs, 12 CEs and 3AAs. The proportions of each CAZymes were compared using Metastats analysis. GH37, CBM50, GT19, GH102, GH4, GH3, GH8, CE6, GH65, CE11, CBM3, GH121, and CBM40 were found to be significantly higher in the W-T2D-RD group ($p<0.05$) than in the P-T2D-RD group; Further LDA analysis found two differentially expressed CAZyme families between groups—CBM50, CE11, GH4, and GT19 in the W-T2D-RD group and CE6, GH121, and GH3 in the P-T2D-RD group (Fig. 3B).



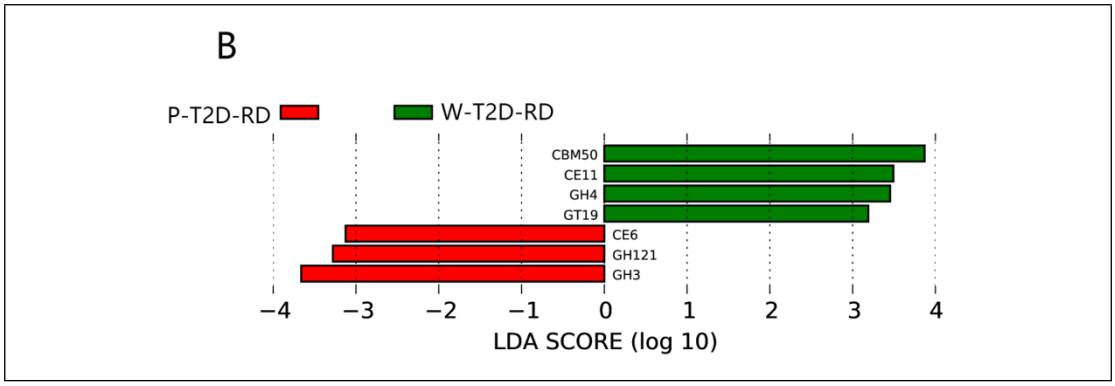


Figure.3 Functional analyses on CAZymes of the gut microbiota. A.Functional categories (level 1) present in the gut metagenome datasets. B. LEfSe analysis of functional differences between groups.

3.2.5 Differential gut microbial KEGG genes, modules, and pathways between P-T2D-RD and W-T2D-RD

Metagenomic sequencing has an inherent advantage in that it allows for the examination of gene content within microbial populations, enabling direct inference of the metabolic capabilities of these populations.To explore the overall functional profiles and potential differences in the functional composition of the gut microbiome of W-T2D-RD group patients versus W-T2D-RD group patients. We performed Kyoto encyclopedia of genes and genomes (KEGG)[27-29] functional module comparisons to uncover potential critical microbiome functions within the two groups,the KEGG database was used to identify unigenes(www.kegg.jp/kegg/kegg1.html). Unigenes matching level 1 and level 2 KEGG functional categories are shown in Fig. 4A. Specifically, the dominant functional categories identified included metabolism (52.4%) , genetic information processing (17.1%), and environmental information processing (13.8%), where the results indicated that the most functionally enriched categories in metabolism were associated with carbohydrate metabolism(22.6%), amino acid metabolism(17.3%), metabolism of cofactors and vitamins(12.8%),energy metabolism(11.2%),nucleotide metabolism(10.7%),glycan biosynthesis and metabolism(6.0%),and lipid metabolism(5.5%).

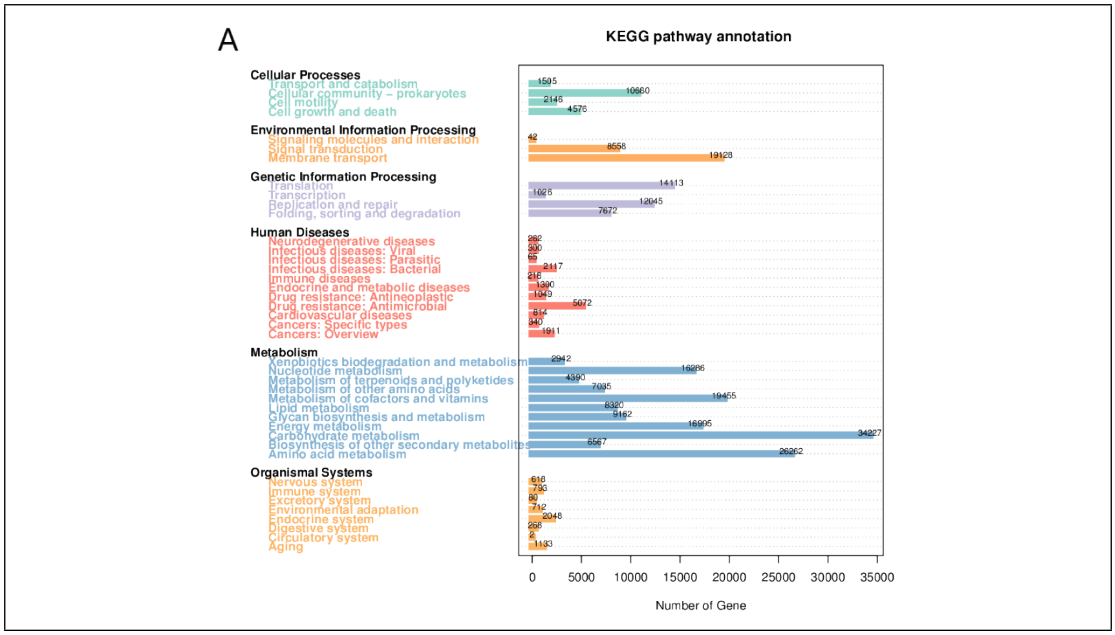


Figure.4 A.Summary of unigenes matched to each KEGG functional categories (level 1 and level 2) present in the gut metagenome datasets.

3.3 The impact of *Lactobacillus johnsonii* on glucose metabolism.

3.3.1 Fasting blood glucose

The fasting blood glucose(FBG) levels of each group of mice are shown in Fig.5A. The CRD group had a non-significant decrease in FBG compared to the C group (P>0.05). Compared to the T2DM-C group, the T2DM-RD-L group had a significant decrease in FBG levels on day 42 of gavage (P<0.05). The T2DM-RD group had lower FBG levels compared to the T2DM-C group (P>0.05). Compared to the T2DM-RD group(P>0.05).

3.3.2 Postprandial blood glucose

The postprandial blood glucose(PBG) levels of each group of mice are shown in Fig. 5B. The PBG levels in the CRD group were higher than those in the C group on day 42 of the rhythmic intervention (P > 0.05). In the T2DM-RD group, PBG levels were higher than those in the T2DM-C group on days 12, 18, 42, and 48 of the rhythmic intervention (P > 0.05). On days 18 and 24 of the gavage, the PBG levels in the T2DM-RD-L group were significantly decreased compared to the T2DM-RD group (P < 0.05).

3.3.3 Insulin

The serum insulin levels of each group of mice are shown in Fig.5C. There were no significant differences in fasting serum insulin levels between the CRD and C groups. There was no significant difference in fasting insulin levels between the CRD group and the C group. Compared to the T2DM-C group, the fasting serum insulin levels in the T2DM-RD-L group were lower (P > 0.05). The fasting serum insulin levels in the T2DM-RD group were also lower than those in the T2DM-C group (P > 0.05). Additionally, the fasting serum insulin levels in the T2DM-RD-L group were lower compared to the T2DM-RD group (P > 0.05).

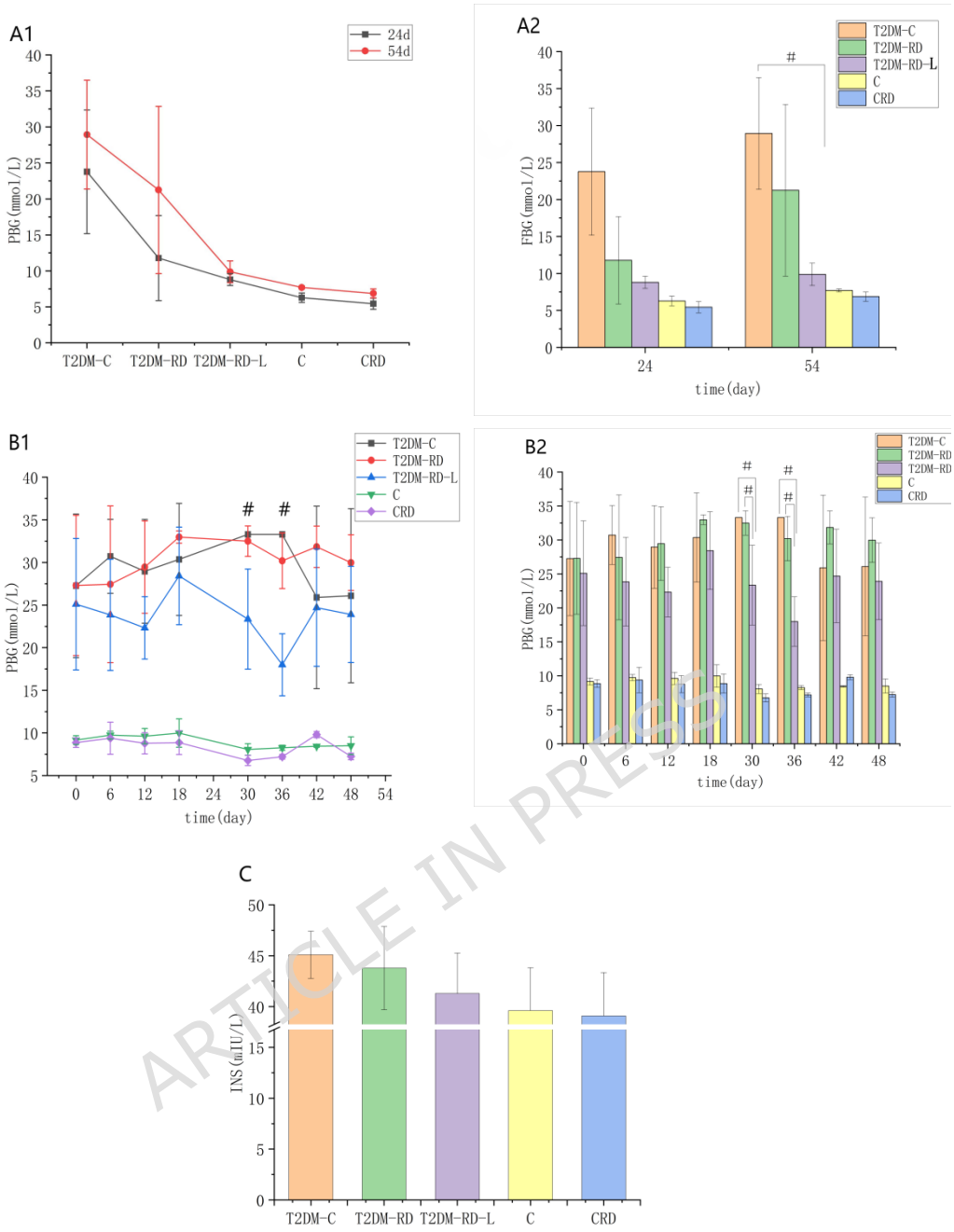


Figure.5 The impact of *L. johnsonii* on glucose metabolism.A(1,2).Results of FBG experiments in mice.B(1,2).Results of PBG experiments in mice.C.Results of INS experiments in mice.

Note: #Significant difference among T2DM-C, T2DM-RD, and T2DM-RD-L groups, P□0.05.

3.4 The impact of *Lactobacillus johnsonii* on lipid metabolism.

3.4.1 Total cholesterol

The fasting total cholesterol (TC) levels of each group of mice are shown in Fig.6A.The TC levels in the CRD group were lower than those in the C group ($P > 0.05$). Compared to the T2DM-C group, the TC levels in the T2DM-RD-L group were significantly reduced ($P < 0.05$). The TC levels in the T2DM-RD-L

group were lower than those in the T2DM-RD group ($P > 0.05$). Compared to the T2DM-C group, the T2DM-RD group had a significant decrease in total cholesterol levels ($P<0.05$).

3.4.2 Triglyceride

The fasting triglyceride (TG) levels of each group of mice are shown in Fig.6B. The TG levels in the CRD group were higher than those in the C group ($P > 0.05$). Compared to the T2DM-C group, the triglyceride levels in the T2DM-RD-L group were lower ($P > 0.05$). There was no significant difference in triglyceride levels between the T2DM-RD and T2DM-RD-L groups. Additionally, the triglyceride levels in the T2DM-RD group were lower than those in the T2DM-C group ($P > 0.05$).

3.4.3 High-density lipoprotein

The HDL levels of each group of mice are shown in Fig.6C. The HDL levels in the CRD group were lower than those in the C group ($P > 0.05$). Compared to the T2DM-C group, the HDL levels in the T2DM-RD-L group were significantly elevated ($P < 0.05$). The HDL levels in the T2DM-RD-L group were also higher than those in the T2DM-RD group ($P > 0.05$). Additionally, the HDL levels in the T2DM-RD group were higher compared to the T2DM-C group ($P > 0.05$).

3.4.4 Low-density lipoprotein

LDL levels of mice in each group were shown in Fig.6D. The LDL levels in the CRD group were higher than those in the C group ($P > 0.05$). The LDL levels in the T2DM-RD-L group were lower than those in both the T2DM-RD and T2DM-C groups ($P > 0.05$). Among these groups, the LDL levels in the T2DM-RD group were higher than those in the T2DM-C group ($P > 0.05$).

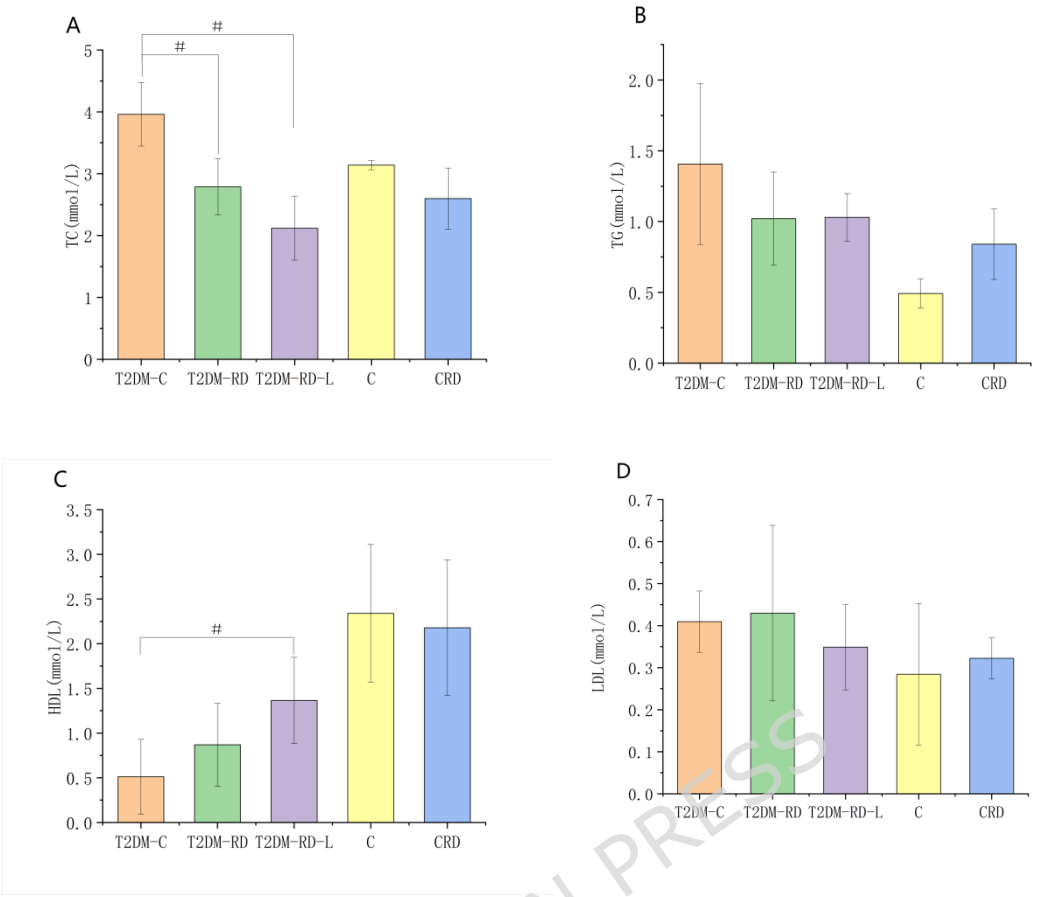


Figure.6 The impact of *L. johnsonii* on lipid metabolism.A. Results of TC experiment in mice.B. Results of TG experiment in mice.C. Results of HDL experiment in mice.D. Results of LDL experiment in mice.
Note: #Significant difference among T2DM-C, T2DM-RD, and T2DM-RD-L groups, P□0.05.

4 Discussion

There is increasing evidence suggesting the important role of gut microbiota in human health, and dysbiosis may contribute to pathological conditions. Recent studies have found a connection between gut microbiota and type 2 diabetes (T2DM). Specifically, imbalances in gut microbiota may lead to abnormal glucose metabolism and increase the risk of developing T2DM. Current research [12, 30] has identified various microbial characteristics. Under normal physiological conditions, *Firmicutes* account for the largest proportion (64%) in the gut microbiota, followed by *Bacteroidetes* (23%), *Proteobacteria* (8%), and *Actinobacteria* (3%). Differences in gut microbiota have been observed between T2DM patients and individuals with normal glucose tolerance [17]. Increased abundance of *Bacteroides caccae, Clostridium hathewayi, Clostridium ramosum, Clostridium symbiosum, Coprococcus eutactus, Clostridia, Collinsella, Desulfovibrio sp., Eggerthella lenta, Escherichia coli, Lactobacillus gasseri, Streptococcus mutans, Lachnospiraceae bacterium, Prevotella, Ruminococcus, Verrucomicrobia, Dorea,* and *Fusobacterium* has been observed in T2DM patients. On the other hand, decreased abundance of

Clostridiales sp. SS3/4, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia intestinalis, Roseburia inulinivorans, Eubacterium eligens, Bacteroides intestinalis, Oscillibacter, Bifidobacterium, Coprococcus, Butyrivibrio, Clostridium hathewayi, Clostridium bolteae, Clostridium symbiosum, Bacteroides, Streptococcus, Bifidobacterium, and *Parabacteroides* has been observed in T2DM patients. The abundance of *Akkermansia muciniphila*, *Streptococcus*, and *Dorea* is controversial, with some studies showing increased expression while others show decreased expression. Additionally, there are differences in gut microbiota between T2DM obese patients, T2DM non-obese patients, obese individuals, and individuals with normal glucose tolerance. For example, the abundance of *Akkermansia muciniphila* [31] was decreased in T2DM and obese patients. Disruption in circadian rhythms is associated with gut microbiota dysbiosis in comparison to individuals with normal sleep-wake cycles [21, 23, 26]. The relative abundance of *Bacteroidetes, Bacteroidia*, and *Spirillum* is decreased, while *Actinobacteria, Firmicutes, Delta-proteobacteria, Desulfuromonadales, Desulfuromonadaceae, Campylobacteriaceae, Corynebacteriaceae, Dorea longicatena*, and *Dorea formicigenerans* is increased. During clinical work, we have observed a portion of T2DM patients who exhibit disrupted circadian rhythms. These individuals typically have poor blood glucose control, possibly due to the nature of their professions (e.g., doctors, train drivers, and truck drivers) that result in irregular eating, resting, and medication schedules, leading to a series of adverse effects. However, there is still a small portion of individuals who are able to achieve good blood glucose control.

Since there have been no reports on gut microbiota in individuals with T2DM combined with circadian rhythm disruption under different blood glucose control conditions, we recruited T2DM patients with circadian rhythm disruption for gut metagenomic sequencing. We discovered that the microbial composition in W-T2D-RD group was more diverse compared to those P-T2D-RD group. This suggests that the abundance of microbiota may be associated with blood glucose control in individuals with circadian rhythm disruption and may influence glucose metabolism. Our study revealed significant differences in the composition of microbial communities at the phylum, genus, and species levels between the two groups. The relative abundance of *Firmicutes* was decreased in the P-T2D-RD group, while the abundance of *Actinobacteria* was increased. These findings are consistent with previous studies on gut microbiota changes in individuals with normal rhythm disruption and in individuals with different blood glucose control states of T2DM (increased relative abundance of *Firmicutes*), but there are also differences (decreased relative abundance of *Actinobacteria*).

Fecal Microbiota Transplantation (FMT) involves transferring healthy gut microbiota from individuals with normal glucose tolerance to the intestines of type 2 diabetes patients using methods such as capsules, gastroscopy, and colonoscopy. This process helps to restore the imbalanced gut microbiota in

T2DM patients and improve glucose metabolism disorders[32, 33]. Previous studies have also demonstrated the efficacy of FMT in alleviating T2DM through numerous animal experiments [34-37]. These studies involved collecting fecal samples from normal mice, processing them, and then transplanting them into T2DM mouse models. FMT was found to potentially improve damaged islets by reducing the secretion of pro-inflammatory cytokines and increasing the secretion of anti-inflammatory cytokines [34]. FMT also showed unique effects in terms of islet regeneration, increased functional β-cell mass, and improved insulin sensitivity [35]. Furthermore, FMT was found to be a safe treatment option, effectively inhibiting weight gain, reducing albuminuria, decreasing local TNF-α expression in the ileum and ascending colon, and improving insulin resistance in mice [36]. Some studies using metagenomic or 16S sequencing have also identified decreased relative abundance of certain bacterial genera in T2DM patients, such as *Blautia wexlerae* [38] and *Akkermansia muciniphila* [31]. Transplanting these strains to T2DM mice has been found to improve blood glucose levels and insulin resistance in these animals.we found an decreased abundance of *L.johnsonii* in P-T2D-RD patients, indicating that lactobacillus johnsonii can be taken as a novel biomarker for the treatment of P-T2D-RD, and that decreased *L.johnsonii* may be attributed to the biological mechanism of P-T2D-RD. *L.johnsonii* has not been used in the treatment of T2DM yet, so we chose *L.johnsonii* for fecal microbiota transplantation. This will help us further understand the pathogenesis of T2DM and discover new therapeutic targets.

we used metagenomics to study the compositional (profiles of microbiota) and functional capabilities (KEGG functional categories,and CAZymes) of the gut microbiomes of P-T2D-RD patients compared to W-T2D-RD. We found that there are significant overlaps and differences in microbial transformation and functional characteristics between the gut microbiota of the two groups.The identified CAZymes enable both P-T2D-RD patients and W-T2D-RD patients to utilize plant material as a significant source of nutrients due to the enzymatic activities of gut microbes.The increased abundance of greenhouse gases in each group indicates the enrichment of different gut microbiota that specialize in utilizing different plant polysaccharides. The overexpression of GHs may lead to an excessive rate of nutrient absorption, thus contributing to the pathogenesis of T2DM with circadian rhythm disruption at the microsystem level.Our results suggest that high carbohydrate intake in the gut may be one of the mechanisms underlying the development of T2DM with circadian rhythm disruption. The pathways and CAZymes identified in our study can serve as a possible biological explanation for hyperglycemia in T2DM with circadian rhythm patients. The significant contraction of carbohydrate metabolism-related enzymes and pathways leading to excessive nutrient absorption resulting in energy surplus increases the risk of obesity and overweight, ultimately leading to poor blood sugar control in T2D with circadian rhythm disruption patients.

Our experimental results show that circadian rhythm disruption leads to

poor PBG control in both T2DM-RD and T2DM-RD-L groups of mice, which is consistent with the previous mention of circadian rhythm disruption causing poor blood glucose control. Furthermore, our experimental results indicate that after administration of *Lactobacillus johnsonii*, postprandial blood glucose in the T2DM-RD-L group of mice improves compared to the T2DM-RD group. Therefore, we consider *L.johnsonii* to be an effective method for improving the poor postprandial blood glucose control caused by circadian rhythm disruption. Proper regulation of insulin levels is crucial for maintaining normal blood glucose metabolism and overall metabolic balance. Previous studies have mentioned that gut microbiota can improve insulin secretion and insulin resistance.Our experimental results indicate that after *L.johnsonii* administration, the insulin levels in the T2DM-RD-L group of mice are lower compared to the T2DM-C group. This may be because the T2DM-C group of mice does not have circadian rhythm disruption and has relatively normal insulin levels, while the administration of *L.johnsonii* leads to a normalization of insulin levels in the T2DM-RD-L group of mice, resulting in a trend of decrease. Dysbiosis of the gut microbiota leads to pathological changes in mice with diabetes and circadian rhythm disruption, including insulin secretion and blood glucose regulation. Our experimental results suggest that gut microbiota transplantation treatment leads to a decrease in fasting insulin levels in patients with type 2 diabetes and circadian rhythm disruption, indicating the potential of microbiota transplantation in improving insulin levels in diabetes. Therefore, *L.johnsonii* may regulate the gut microbiota of diabetic mice, restoring partial functionality to these physiological processes and improving blood glucose control and insulin secretion in the T2DM-RD-L group of mice.

In conclusion, these results suggest that *L.johnsonii* improves blood glucose control and insulin levels by regulating the gut microbiota and restoring partial functionality to physiological processes such as insulin secretion and blood glucose regulation in diabetic mice. Although most studies on gut microbiota transplantation for type 2 diabetes treatment have been conducted on diabetic mouse models, as mentioned in the introduction, there have been studies transplanting fecal samples from healthy individuals, after purification, into the intestines of diabetic patients, which have shown positive effects such as weight loss, decreased blood glucose levels, and reduced insulin resistance. Although there have been limited studies on single strain microbiota transplantation, we believe that *L.johnsonii* can have positive implications for diabetes management in a clinical setting. Further research and clinical trials are needed to confirm the applicability and efficacy of these results in patients with type 2 diabetes.

The gut microbiota transplantation also has a positive effect on lipid metabolism. Our experimental results indicate that after *L.johnsonii* administration, the high-density lipoprotein (HDL) levels in the T2DM-RD-L group are improved compared to the T2DM-C group, suggesting that *L.johnsonii* administration may have some benefits in improving HDL metabolism in the

T2DM-RD-L group of mice. However, the difference is not significant compared to the T2DM-RD group, which may be attributed to the positive effects of forced exercise and intermittent fasting due to sleep disturbance in the T2DM-RD group. The low-density lipoprotein (LDL) levels in the T2DM-RD-L group are decreased compared to both the T2DM-RD and T2DM-C groups, indicating that *L.johnsonii* administration may have some benefits in improving LDL metabolism in T2DM mice. Total cholesterol and triglyceride levels are important indicators of lipid metabolism, and studies have shown that the T2DM-RD-L group has improved total cholesterol and triglyceride levels compared to the T2DM-C group, indicating that *L.johnsonii* helps improve total cholesterol and triglyceride levels in T2DM mice.

In summary, *L.johnsonii* administration improves certain lipid metabolism indicators (such as HDL levels, total cholesterol levels, triglyceride levels) in the T2DM-RD-L group compared to the T2DM-C group. Therefore, we believe that *L.johnsonii* administration can help improve lipid metabolism in T2DM mice with disrupted circadian rhythms, manifested by increased HDL levels, decreased LDL levels, and improved total cholesterol and triglyceride levels. This may be attributed to the effects of *L.johnsonii* on the gut microbiota, which in turn regulates the balance of lipid metabolism in the body. *L.johnsonii* may improve the composition of the gut microbiota, promote the activity of lipid metabolism-related enzymes, or influence the absorption and utilization of lipids in the intestines, thus improving lipid metabolism.

Through animal experimentation, we have demonstrated the potential benefits of *L.johnsonii* in improving blood lipid levels in diabetic mice. Furthermore, several studies have shown that the transplantation of fecal matter from individuals with normal glucose tolerance to diabetic patients can significantly improve their blood lipid levels. Based on the potential of *L.johnsonii* in improving lipid metabolism in diabetic mice, we believe that it may also have the potential to improve blood lipid levels in diabetic patients. However, this hypothesis needs to be verified through subsequent clinical trials. Although we have not yet applied this to clinical diabetic patients, these findings provide new prospects for the clinical application of *L.johnsonii*.

Our study has several limitations. First, the sample size was small, and we did not include female patients or individuals with normal glucose tolerance. Second, in the animal experiment, we did not perform glucose tolerance tests and insulin tolerance tests on the mice due to considerations of the number of experimental animals and their condition. As a result, we were unable to evaluate their pancreatic function. Third, in the animal experiment, we found some improvements in certain glucose and lipid metabolism indicators in the T2DM-RD group compared to the T2DM-C group, which could be attributed to the beneficial effects of forced exercise. However, this was not further validated. Fourth, we did not conduct further metagenomic sequencing and untargeted metabolomics sequencing on the fecal samples of the mice in each group to validate or explore the relationship between the gut microbiota and metabolites.

5.Conclusion

In individuals with type 2 diabetes mellitus (T2DM) and disrupted circadian rhythm, there are notable differences in the composition of gut microbiota between those with well-controlled blood glucose levels and those with poor blood glucose control.Transplantation of *Lactobacillus johnsonii* has shown significant improvements in glucose metabolism and lipid metabolism levels in mice with disrupted circadian rhythm and T2DM. This suggests that *Lactobacillus johnsonii* may have potential value in the treatment of T2DM.

Author contributions:

Wang Defeng,Wang Zhen, and Yang Yan conceived and designed the project. Yang Yan, Shen Hongxia and Sun Li collected samples. Yang Yan, Shi Zhenhong, Mei Xianghui and Wu Na conducted animal experiments.Yang Yan wrote the manuscript. Wang Defeng and Wang Zhen reviewed and edited the manuscript. All authors made substantial contributions and approved the final version of the manuscript.

Data Availability:

The DNA sequencing data is available in NCBI Sequence Read Archive (SRA) under the Accession Number SUB14302900(PRJNA1087526). https://submit.ncbi.nlm.nih.gov/subs/sra/SUB14302900/overview.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Hebei University of Engineering affiliated hospital. The patients/participants provided their written informed consent to participate in this study.This animal experiments was approved by the Institutional Animal Care and Use Committee of Hebei University of Engineering affiliated hospital (IACUC-Hebeu-2023-0006).

Conflict of interest

The authors declare no competing interests.

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