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Therapeutic Effects of Intermittent Fasting Combined with SLBZS and Prebiotics on STZ-HFD-Induced Type 2 Diabetic Mice

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Purpose: This study aims to assess the therapeutic potential of combining Shen-Ling-Bai-Zhu-San (SLBZS) or prebiotics with intermittent fasting (IF) in type 2 diabetes mellitus (T2DM) mice and to investigate the synergistic effects and underlying mechanisms.

Methods: Type 2 diabetic mouse models were induced using high-fat diet (HFD) and streptozotocin (STZ), followed by IF treatment. Mice were then grouped for combined therapy with different doses of SLBZS and prebiotics. Fasting blood glucose (FBG) levels, body weight variations, and oral glucose tolerance tests were assessed to elucidate metabolic alterations. The hepatic and renal parameters were evaluated to determine systemic changes in T2DM mice, while the insulin levels were quantified by ELISA to assess glucose homeostasis. Gut microbiota alterations were examined via 16S rRNA sequencing. Alterations of the genes in relevant signaling pathways were analyzed using RT-qPCR.

Results: IF improved FBG, body weight, insulin levels, and other diabetes indicators. Combined IF with SLBZS or prebiotics yielded similar effects. Furthermore, it ameliorated dyslipidemia and mitigated hepatic and renal parameters in T2DM mice. Pancreatic tissue histopathology showed islet cell restoration post-intervention. IF therapy reduced the abnormally elevated *GSK-3β* gene expression and increased the abnormally reduced *GLUT2* genes. Further analysis indicated that the combination of IF with prebiotics and high doses of SLBZS upregulated the expression of the *INSR* and *IRS1* genes. Gut microbiota analysis revealed restored diversity and structure, with notable changes in specific bacterial families. At the family level, the contents of *Akkermansiaceae* and *Bifidobacteriaceae* were restored. Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2) analysis suggested metabolic pathway alterations.

Conclusion: IF improved type 2 diabetic symptoms, with combined SLBZS and prebiotics showing similar effects. IF with high concentration of SLBZS and prebiotics doses upregulated the *INSR* and *IRS1* genes and had superior effects on gut microbiota compared to IF alone.

Keywords: [type 2 diabetes mellitus, intermittent fasting, prebiotics, Shen-Ling-Bai-Zhu-San]

Introduction

Diabetes, a metabolic disorder characterized primarily by hyperglycemia, is widely prevalent worldwide. According to the 10th edition of the IDF Diabetes Atlas, approximately 537 million adults (aged 20–79 years) globally were living with diabetes in 2021,[1] and this number is predicted to rise to 643 million by 2030 and 783 million by 2045. Of these cases, over 90% are diagnosed as type 2 diabetes,[2] which is influenced by various factors including genetics, lifestyle, and environmental factors. Due to factors such as increasing obesity rates, sedentary lifestyles, and poor dietary habits, the incidence of type 2 diabetes is rising among children, adolescents, and young adults.[3,4] The main causes of type 2 diabetes include insulin resistance and pancreatic β-cell dysfunction. Insulin resistance leads to decreased sensitivity of pancreatic cells, diminished glucose uptake, and impaired glycogen synthesis, resulting in hyperglycemia. To counteract high blood glucose levels, the body increases insulin secretion, but prolonged hyperglycemia damages pancreatic β-cell function, making reversal difficult. Thus, the development of type 2 diabetes is closely associated with insulin resistance

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and pancreatic β-cell dysfunction.[5] If blood glucose levels are not effectively controlled, diabetes can also lead to damage to the liver and kidneys in the organism.[6,7]

Intermittent fasting (IF) is a relatively popular dietary approach in recent years, involving cyclic periods of zero or reduced calorie intake alternated with normal calorie consumption, aimed at preventing and treating metabolic diseases.[8] The three common IF patterns include alternate day fasting (ADF), the 5:2 diet, and time-restricted eating (TRE).[9–11] Clinical trial results have demonstrated that IF can effectively reduce body weight in obese individuals, improve insulin resistance and lipid metabolism, and decrease the incidence of diabetes and cardiovascular diseases.[12] By extending fasting periods, IF maximizes the oxidation of fatty acids and ketones, rather than relying on glucose as the primary energy source.[13] Additionally, IF can induce reshaping of the gut microbiota,[5] with alterations in the microbiota closely associated with fat loss, weight reduction, and decreased blood glucose levels.[14,15]

The human gastrointestinal tract harbors a complex and diverse microbial community known as the gut microbiota, which forms a symbiotic relationship with the human body.[16,17] These microorganisms influence the physiological metabolism of the host through direct interactions or metabolic products.[18–20] The application of high-throughput sequencing technologies has revealed the relationship between gut microbiota composition and metabolic diseases such as obesity, non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes mellitus (T2DM). Studies have found that newly diagnosed T2DM patients exhibit reduced gut microbiota diversity, with decreased abundance of butyrate-producing bacteria such as Bifidobacterium and Akkermansia, and increased levels of Dorea, leading to the occurrence and maintenance of insulin resistance.[21] Additionally, various gut microbiota metabolites, such as short-chain fatty acids, trimethylamine (TMA), and tryptophan derivatives, are closely associated with the pathogenesis of T2DM.[22,23] In regulating gut microbiota, prebiotics play a beneficial role and are defined by the international nutrition community as dietary fibers that promote the growth and proliferation of beneficial gut bacteria. They can modulate the structure and function of the gut microbial community, exerting positive effects on digestion, absorption, metabolism, and immunity in the human body.[24–26]

Traditional Chinese medicine (TCM) has a long history and offers diverse approaches to treating diabetes. Despite the effectiveness of medications like metformin in improving insulin resistance and reducing blood glucose levels, some individuals experience gastrointestinal discomfort and other side effects. Chinese herbal medicine contains various components, such as polysaccharides, polyphenols, and amino acids, which can comprehensively target multiple pathways to improve pathological conditions and achieve therapeutic effects. Shen-Ling-Bai-Zhu-San (SLBZS), originating from the Song Dynasty, consists of ten medicinal herbs that collectively tonify qi and invigorate the spleen. These kinds of herbs belong to the category of medicinal and edible plants, exhibiting high safety profiles suitable for daily consumption and nourishment. Research has shown that ginseng and Atractylodes macrocephala not only modulate the gut microbiota but also meet the nutritional needs of the intestines.[27,28] Furthermore, SLBZS improves non-alcoholic fatty liver disease (NAFLD) induced by a high-fat diet by increasing the abundance of beneficial gut microbiota,[29] and it also treats ulcerative colitis (UC) and T2DM.[30]

In the specific practice of treating type 2 diabetes, IF, while effective in reducing blood glucose levels and improving insulin resistance, often requires an extended treatment duration. However, many patients struggle to adhere to IF due to lifestyle changes and the hunger associated with prolonged fasting periods. Given the known effects of SLBZS and prebiotics on blood glucose regulation and modulation of the gut microbiota, this study aims to explore the therapeutic effects of IF combined with prebiotics and SLBZS in high-fat diet and streptozotocin (HFD-STZ) induced type 2 diabetic mice. Through analyzing relevant gene expression and gut microbiota, the study seeks to elucidate the potential mechanisms, aiming to provide a reference for optimizing and enhancing the effectiveness of IF in the intervention and treatment of type 2 diabetes.

Material and Methods

Chemicals and Reagents

Streptozotocin was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). The high-fat diet consisted of 78.8% basal diet, 1% cholesterol, 10% egg yolk powder, 10% lard, and 0.2% bile salt, was obtained from

SPF Biotechnology Co., Ltd (Beijing, China). Chinese herbal medicines were obtained from Beijing Tongrentang Co., Ltd., China. Prebiotics were provided by Beijing Yunyi International Technology Co., Ltd (Beijing, China). The Enzyme-linked Immunosorbent Assay (ELISA) Kit was provided by Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). The RNA Easy Fast Animal Tissue Cell Total RNA Extraction Kit was provided by Tiangen Biotech Co., Ltd (Beijing, China). The RevertAid First Strand cDNA Synthesis Kits were purchased from Thermo Fisher Scientific Inc (Beijing, China).

Preparation of SLBZS and Prebiotics

SLBZS is composed of 10 traditional Chinese medicinal herbs, including Panax ginseng C. A. Mey., Poria cocos (Schw). Wolf, Atractylodes macrocephala Koidz, Dioscorea opposita Thunb., Dolichos lablab L., Coix lacryma-jobi L., Glycyrrhiza uralensis Fisch, Platycodon autumnalis Decne., Nelumbo nucifera Gaertn and Amomum villosum Lour. The composition ratio of the medicinal herbs is shown in Table 1. The herbs were soaked in distilled water for 1 hour, then boiled in 500 mL of distilled water for 40 minutes, and this process was repeated twice. The resulting solution was centrifuged and filtered, then concentrated to 0.875 g/mL and 1.75 g/mL respectively. The main component of the prebiotics is dietary fiber, which was dissolved in distilled water and different concentrations of SLBZS solution to a final concentration of 1 g/mL.

Animal Experiments and Grouping

A total of 63 Male SPF C57BL/6J mice, aged 7 weeks, were procured from the SPF (Beijing) Biotechnology Co., Ltd (Beijing, China). Animal experiments were approved by the Experimental Animal Ethics Committee of Beijing University of Chinese Medicine, with the approval number BUCM-2023041304-2020. Follow the rules of 3R during the animal experiments, the experimental mice were housed under suitable pathogen-free (SPF) barrier conditions, with a temperature of 22~26 °C, relative humidity of 50% ~ 60%, well-ventilated environment, and a light-dark cycle. After one week of adaptation, the mice were randomly divided into two groups: the control group (CTRL group, n=9) fed with a normal diet, and the high-fat diet group. The control group mice were fed with a standard diet, while the high-fat diet group received a high-fat diet. After three months, the high-fat diet group mice were intraperitoneally injected with 80 mg/kg of streptozotocin (STZ) sodium citrate buffer, followed by a second injection one week later, preceded by a 16-hour fast. Mice with fasting blood glucose (FBG) levels exceeding 11.1 mmol/L one week later were considered as type 2 diabetic model mice.[31] All animals survived and developed diabetes following the STZ treatment. After then, these type 2 diabetic model mice were randomly divided into six groups (n=9) as the model group (Model), IF group (IF), IF + SLBZS-L group (IF-L), IF + SLBZS-H group (IF-H), IF + prebiotics group (IF-Pre), and the IF + SLBZS-H + prebiotics group (IF-H-Pre). Among them, the IF-L group, IF-H group, and IF-H-Pre group received low dose (5 g/kg/day), high dose (10 g/kg/day) of SLBZS respectively. The estimated dose of prebiotics was based on in vivo models without adverse

Table 1 Composition of Shen-Ling-Bai-Zhu-San (SLBZS) Used in This Study (One Dose)

Name in Chinese	Plant	Part used	Amounts (g)
Ren Shen	*Panax ginseng* C.A.Mey.	Root and Rhizoma	10
Fu Ling	*Poria cocos* (Schw.)Wolf	Sclerotium	10
Bai Zhu	*Atractylodes macrocephala* Koidz	Rhizoma	10
Shan Yao	*Dioscorea opposita* Thunb.	Rhizoma	10
Bai Bian Dou	*Dolichos lablab* L.	Seed	7.5
Lian Zi	*Nelumbo nucifera* Gaertn.	Seed	5
Yi Yi Ren	*Coix lacryma-jobi* L.	Kernel	5
Gan Cao	*Glycyrrhiza uralensis* Fisch	Root and Rhizoma	10
Jie Geng	Platycodon autumnalis Decne.	Root	5
Sha Ren	*Amomum villosum* Lour.	Fruit	5

Table 2 The Experimental Grouping in This Study

Group	Intermittent fasting (16+8)	Low dose of SLBZS (5g/kg)	High dose of SLBZS (10g/kg)	Prebiotics (2g/day)
CTRL				
Model				
IF	O			
IF-L	O	O		
IF-H	O		O	
IF-Pre	O			O
IF-H-Pre	O		O	O

Note: The entries marked with "O" indicate that this group received the corresponding intervention.

effects and is equivalent to a dose of 30 g of prebiotics for an adult of 60 kg. The IF-Pre group and IF-H-Pre group received 0.2 g/day of prebiotics. The grouping is shown in Table 2. The CTRL group mice and Model group mice were freely fed standard feed and water, while the mice in other groups underwent IF for 32 consecutive days.

Intermittent Fasting

The IF method used in this experiment is Time-Restricted Eating (TRE), which restricts daily eating to an 8-hour window, with the remaining 16 hours designated for fasting. In the experimental setup, mice subjected to IF were allowed to freely eat from 9:00 to 17:00 each day without restricting their calorie intake. However, from 17:00 to 9:00 the following day, the mice were subjected to a fasting regimen (Figure 1A). Throughout the duration of the experiment, mice were provided unrestricted access to water.[12]

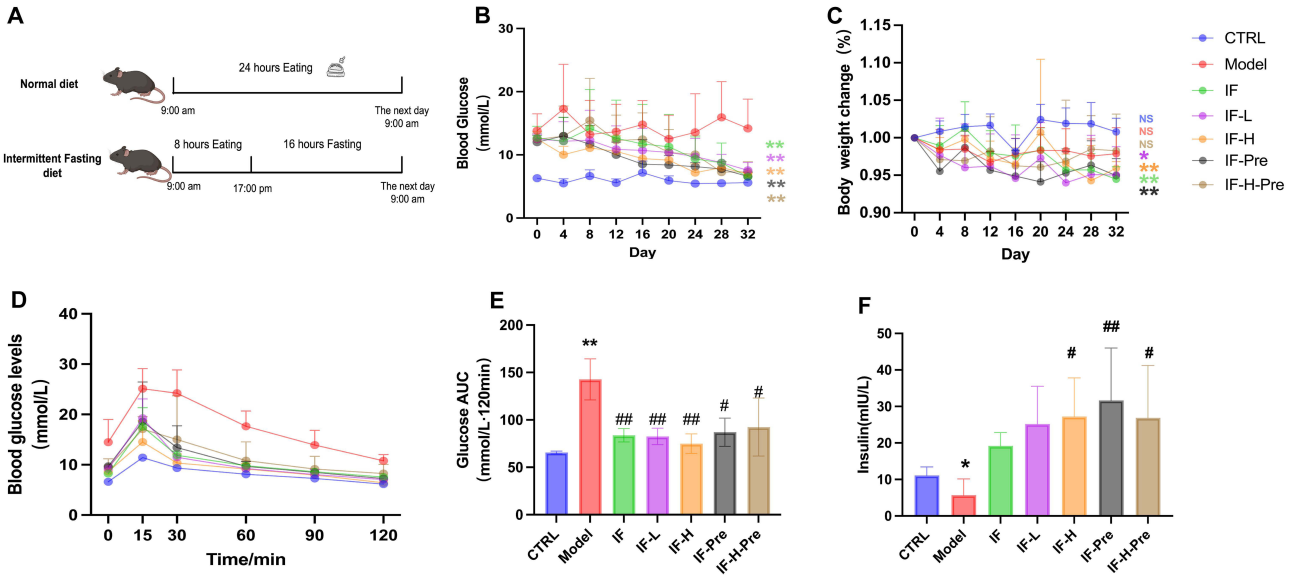

Figure 1 Improvement of fasting blood glucose, body weight changes, glucose tolerance, and insulin levels in type 2 diabetic mice with intermittent fasting combined with SLBZS and prebiotics. CTRL: Control group; Model: Model group; IF: intermittent fasting group; IF-L: IF combined with low concentration of Shen-Ling-Bai-Zhu-San (SLBZS) group; IF-H: IF combined with high concentration of SLBZS group; IF-Pre: IF combined with prebiotics group (IF-Pre); IF-H-Pre: IF combined with high concentration of SLBZS and prebiotics group (IF-H-Pre). (**A**) Feeding patterns of mice under normal diet and intermittent fasting. (**B**) Changes in fasting blood glucose (FBG) levels of mice in each group during the experiment. (**C**) Percentage change in body weight of mice in each group based on the weight recorded on the first day of the experiment. (**D**) Recovery of glucose tolerance in mice after intermittent fasting combined with SLBZS and prebiotics treatment. (**E**) Analysis of area under the curve (AUC) for glucose tolerance in each group. (**F**) Quantification of serum insulin levels in mice from each group using the ELISA assay. $^{*}P < 0.05$ and $^{**}P<0.01$ vs CTRL group; $^{\#}P < 0.05$ and $^{\#\#}P<0.01$ vs Model group.The colors of lines and symbols correspond to the grouping colors.

Oral Glucose Tolerance Test

Following a 32-day IF intervention, oral glucose tolerance tests (OGTT) were conducted on mice. A 20% glucose solution was administered via oral gavage to mice fasted for 12 hours at a dosage of 2 g/kg. Blood samples were collected from the tail vein at 0, 15, 30, 60, 90, and 120 minutes post-gavage, and blood glucose levels were measured using a glucometer. The area under the curve (AUC) was calculated to assess changes in blood glucose levels over time.[4,32]

Data and Sample Collection

Throughout the experiment, the body weight and fasting blood glucose of the mice were recorded at fixed times every four days. Following the conclusion of the experiment, mice were fasted for 12 hours, and fecal samples were collected and stored at −80 °C in an ultra-low temperature refrigerator. Blood samples were obtained from the mice using the retro-orbital blood collection method. At the end of the experiment, all mice were euthanized, and their pancreas were collected for histopathological examination.

Analysis of Pancreatic Tissue Morphological Changes in Mice

The mouse pancreatic tissue was extracted, with a portion fixed in 4% paraformaldehyde and another portion stored in a −80 °C freezer. Tissue samples fixed in 4% paraformaldehyde were dehydrated, embedded in paraffin, and sectioned into 2–3 μm slices. The sections were baked at 65°C for 60 minutes, deparaffinized, stained with hematoxylin and eosin (HE), observed under a microscope, photographed, and analyzed for morphological changes of the pancreatic tissue.

Hepatic and Renal Parameters

After collecting blood samples from mice, the upper layer of serum was obtained following centrifugation and allowed to stand. Hepatic and renal parameters was then conducted using an automatic biochemistry analyzer to measure lipid-related parameters including cholesterol (CHO), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C), as well as liver function indicators such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Kidney function indicators such as uric acid (UA), creatinine (CRE), and blood urea nitrogen (BUN) were also assessed.

Insulin Levels in Mice Were Assessed with ELISA

The insulin (INS) content in mouse serum was detected using a competitive assay method. Samples were added to wells pre-coated with antibodies, followed by the addition of biotinylated detection antigens. Incubation at 37°C for 30 minutes facilitated the formation of immune complexes through competitive binding between the two components and solid-phase antibodies. After washing away unbound biotinylated antigens with PBST, avidin-HRP was added and incubated at 37°C for 30 minutes, allowing for the binding of avidin-HRP to biotinylated antigens. Subsequent washing removed unbound avidin-HRP, and the bound HRP catalyzed TMB to form a blue color, which turned yellow upon acidification. Absorbance peaks were measured at 450nm wavelength, and absorbance values were inversely correlated with antigen concentrations in the samples. Sample concentrations were calculated based on the standard curve.

16S rRNA Sequencing of the Gut Microbiota

Mouse fecal samples were collected and stored at −80°C freezer until analysis. Total DNA was extracted from the samples. The primers (Forward: ACTCCTACGGGAGGCAGCA; Reverse: GGACTACHVGGGTWTCTAAT) targeting conservative regions were designed. Sequencing adapters were added to the ends of the primers, followed by PCR amplification. The resulting products were purified, quantified, and normalized to form sequencing libraries. Qualified libraries underwent quality control and were sequenced using Illumina NovaSeq 6000. Raw image data files obtained from high-throughput sequencing were processed through base calling and converted into raw sequencing reads. Final valid data were obtained after quality filtering and DADA2 denoising. Further analyses including alpha diversity, beta diversity, significant species difference analysis, correlation analysis, and functional prediction were conducted using bioinformatics methods to explore differences among samples.

Table 3 Gene-Specific Primers Used in RT-qPCR

Gene	PCR primer
β-actin	Forward, 5′-GCCTTCCTTCTTGGGTATGG-3′
	Reverse, 5′-GCACTGTGTTGGCATAGAGG −3′
INSR	Forward, 5′-CAGCCACCACACTCACACTTCC-3′
	Reverse, 5′-CAGCCACACTGCACCTCTCATC-3′
IRS1	Forward, 5′-AGGAGAGTGGTGGAGTTGAGTTG-3′
	Reverse, 5′-AGAAGAAGAGGCTGTGGAGGATG-3′
GSK-3β	Forward, 5′-AAGGACTCACCAGGAGCAGGAC-3′
	Reverse, 5′-CAGGTGTGTCTCGCCCATTTGG-3′
GLUT2	Forward, 5′-GGCTGTCTCTGTGCTGCTTGTG-3′
	Reverse, 5′-AGCCAGTGCCAGAGCCGTAG-3′

RNA Extraction and Real-Time Quantitative PCR

Twenty milligrams of mouse liver tissue were homogenized in 350μL of RNA lysis buffer (RLA) using a low-temperature tissue homogenizer. Subsequently, 10μL of proteinase K was added, and total RNA was extracted from the mouse liver tissue according to the instructions provided in the RNA Easy Fast Animal Tissue and Cells Total RNA Extraction Kit. The quantity and purity of RNA were assessed. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit, following the manufacturer's instructions. Real-time PCR analysis of the resulting cDNA was performed using the SYBR® Premix Ex Taq™ kit. Data were analyzed using the ΔΔCT method with the actin gene as the reference. Primers were custom-designed and synthesized by Shanghai Shenggong Company according to standard protocols. Primer sequences are listed in Table 3.

Statistical Analysis

The experimental results are expressed as mean ± SD. Inter-group comparisons were assessed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test. A significance level of $P < 0.05$ indicates a significant difference, while $P < 0.01$ indicates an extremely significant difference. Data were analyzed using the SPSS v28.0.1.1, and plots were generated using Prism v9.5.1. The original data from the 16S rRNA amplification of intestinal microbiota were processed for redundancy, dividing operational taxonomic units at 97% similarity, upon which species composition and diversity analyses were conducted.

Results

Changes in Fasting Blood Glucose, Body Weight, Insulin Levels, and Glucose Tolerance in STZ-HFD Induced Mice

The analysis of the collected data reveals that IF significantly reduces FBG levels in type 2 diabetic mice ($P<0.01$), as shown in Figure 1B. Furthermore, combining IF with different concentrations of SLBZS or prebiotics also effectively reduces FBG levels in type 2 diabetic mice ($P<0.01$), indicating a favorable regulatory effect of IF and its combination with SLBZS and prebiotics on FBG levels in these mice. However, when comparing IF alone with the combined groups of SLBZS and prebiotics, there is no significant difference observed in lowering FBG levels in type 2 diabetic mice. This indicates that IF alone exerts a relatively strong intervention effect on FBG levels, suggesting its efficacy in FBG regulation.

Subsequently, we analyzed the changes in body weight among the groups. The results are depicted in Figure 1C, showing that IF also significantly reduces body weight in type 2 diabetic mice ($P < 0.01$). Analyzing the rate of body weight change in type 2 diabetic mice during the experiment, it was observed that, on the basis of IF, the combined use of high-dose of SLBZS ($P < 0.01$) was more effective in reducing body weight compared to the combined use of low-dose SLBZS ($P < 0.05$). However, no significant changes in body weight were observed in mice from the group combining IF with high-concentration SLBZS and prebiotics.

Glucose tolerance tests were conducted on type 2 diabetic mice post-treatment, followed by the analysis of experimental results. As depicted in Figure 1D–E, the area under the curve of OGTT for type 2 diabetic mice induced STZ-HFD was significantly higher than that of the CTRL group ($P < 0.01$). Compared with the Model group and IF-Pre group, the IF-H-Pre group exhibited differences ($P<0.05$), and there was a remarkably significant difference between the IF group and the IF group combined with high and low concentrations of SLBZS ($P<0.01$). These findings suggest that the combined use of SLBZS at different concentrations significantly enhances glucose tolerance in STZ-HFD-induced type 2 diabetic mice on the basis of IF.

Insulin content in collected mouse blood samples was measured, and the results are shown in Figure 1F. In type 2 diabetic mice, insulin resistance led to elevated insulin levels in the blood. The serum insulin levels in the IF-Pre group mice were significantly higher than those in the CTRL group ($P<0.05$), while no significant differences in serum insulin levels were observed between the IF group and the IF group combined with high and low concentrations of SLBZS compared to the CTRL group, indicating that IF may partially ameliorate insulin resistance in STZ-HFD-induced type 2 diabetic mice. Furthermore, the combined use of SLBZS at different concentrations achieved similar effects on this basis.

In short, IF can regulate fasting blood glucose, reduce weight, and alleviate impaired glucose tolerance and insulin resistance in STZ-HFD-induced type 2 diabetic mice. The combined use of SLBZS and prebiotics on the basis of IF has the similar effect.

The Effects of if Combined with Prebiotics or SLBZS on the Morphological Changes of Pancreatic Tissue in Type 2 Diabetic Mice

The H&E staining results of pancreatic tissue sections were presented in Figure 2A–G. In the same field of view, it was evident that pancreatic islet cells in the CTRL group mice exhibit regular elliptical shapes, compact arrangement, and uniform cytoplasmic abundance. In comparison to the CTRL group mice, the pancreatic islets in the Model group mice seemed to be atrophy and deformation, with unclear edges and disrupted morphology. The islet cells were observed to be disordered and scattered, indicating damage to pancreatic islet cells in type 2 diabetes induced by a high-fat diet combined with streptozotocin.

Three mice were randomly selected from each group, and the area of any nine pancreatic islets in each mouse was calculated and analyzed, as shown in Figure 2H. Compared to the CTRL group, the pancreatic islet area in mice of the HFD-STZ-induced type 2 diabetes model group significantly decreased ($P < 0.01$). Furthermore, compared to the model group mice, the pancreatic islet area in mice of the intermittent fasting group and the intermittent fasting combined with high-dose of SLBZS group significantly recovered ($P < 0.01$). The pancreatic islet area in mice of the remaining groups showed an increasing trend compared to the Model group. The morphology of pancreatic islets in mice from each group partially recovered, with clearer shapes.

The Impact of Intermittent Fasting Combined with Either Prebiotics or SLBZS on the Hepatic and Renal Parameters of T2DM Mice

Analysis of blood examination results in mice revealed that CHO and LDL-C levels were significantly higher in T2DM mice induced by STZ-HFD compared to the CTRL group ($P<0.01$), indicating a certain degree of lipid metabolism abnormality in the Model group mice with T2DM. Additionally, the renal function-related indicators such as BUN, UA, and CRE, as well as the liver function-related indicators such as ALT and AST, were significantly higher in mice of the Model group compared to the CTRL group ($P<0.01$), suggesting a certain degree of liver and kidney dysfunction in the T2DM mice (Figure 3).

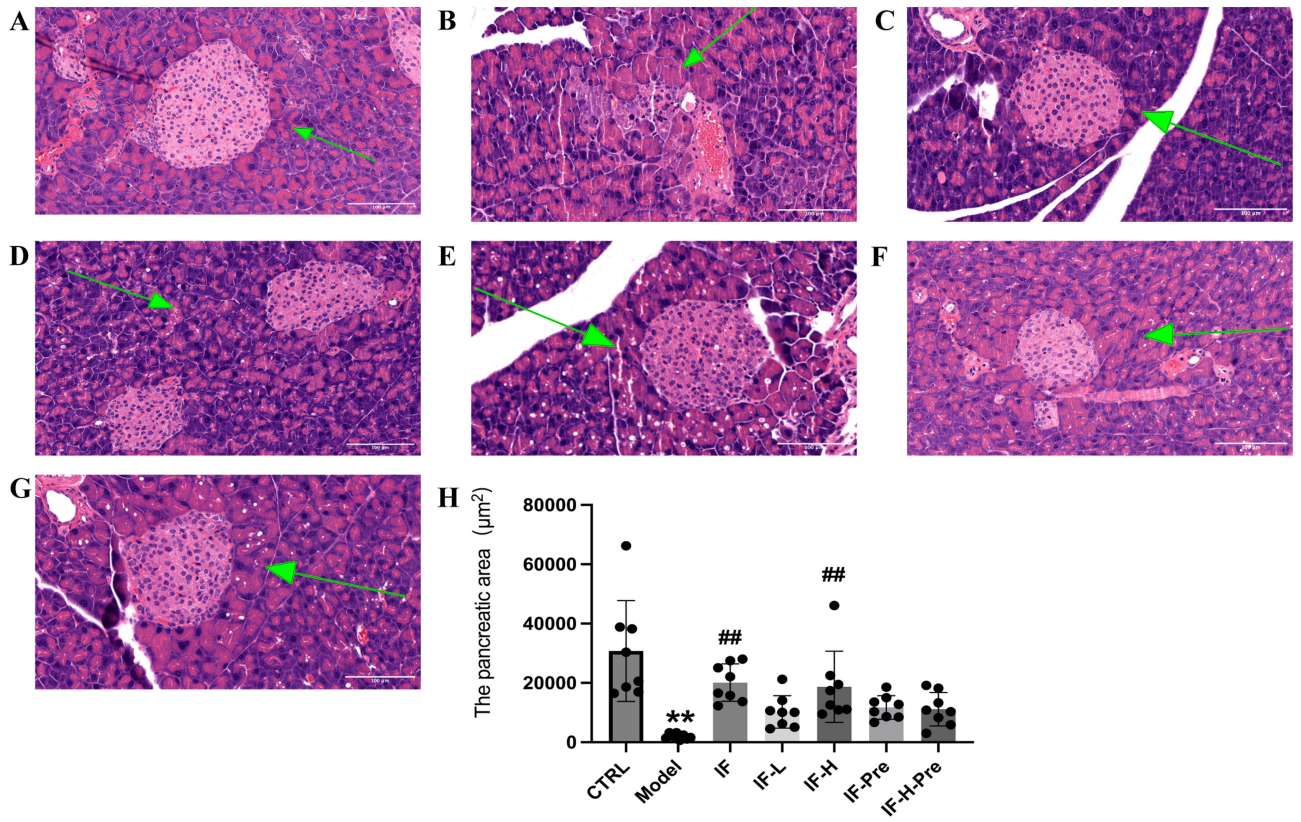

Figure 2 The figure illustrates the H&E staining results of pancreatic sections from each group of mice to analyze the pancreatic changes. Green arrows indicate pancreatic islet cells. Scale bar = 100 μm. (**A**) CTRL: Control group; (**B**) Model: Model group; (**C**) IF: Intermittent fasting group; (**D**) IF-L: IF combined with low concentration of Shen-Ling-Bai-Zhu-San (SLBZS) group; (**E**) IF-H: IF combined with high concentration of SLBZS group; (**F**) IF-Pre: IF combined with prebiotics group (IF-Pre); (**G**) IF-H-Pre: IF combined with high concentration of SLBZS and prebiotics group (IF-H-Pre). (**H**) Statistical analysis of pancreatic islet area in mice from each group (n=3). $^{**}P<0.01$ vs CTRL group; $^{\#\#}P<0.01$ vs Model group.

Following intermittent fasting treatment, the above-mentioned abnormal indicators significantly decreased ($P<0.05$), indicating that intermittent fasting can restore the lipid metabolism abnormalities and liver and kidney dysfunction present in T2DM mice. Furthermore, when combined with different doses of SLBZS and prebiotics, they demonstrated similar effects, significantly reducing the aforementioned abnormal indicators.

The Effects of if Combined with Prebiotics or SLBZS on the Changes of the Diversity and Composition of Gut Microbiota in T2DM Mice

To investigate the potential role of gut microbiota in the treatment of type 2 diabetes, mouse fecal samples were collected for 16S rRNA sequencing of gut microbiota. Shannon index curve and Rank-abundance curve based on the number of operational taxonomic units (OTU) of different samples showed that the sequencing depth and detection of sample diversity in this study were deemed adequate for reference, ensuring reliable sequencing results (Figure 4A–B). The results of α-diversity analysis showed that the Shannon index and Simpson index of mice in the Model group were lower than those of mice in the CTRL group ($P<0.01$) (Figure 4C–D), indicating a significant decrease in gut microbiota alpha diversity in type 2 diabetic mice. Through the IF intervention, the Simpson index of type 2 diabetic mice was restored ($P<0.05$), and the Shannon index of type 2 diabetic mice was also restored by the combined use of prebiotics during IF ($P<0.05$).

Principal component analysis (PCA) and principal coordinates analysis (PCoA) were conducted on the mouse gut microbiota at the species level. The results revealed that after treatment with intermittent fasting combined with SLBZS and prebiotics, the distance between the gut microbiota of mice and the CTRL group mice decreased, indicating a more similar gut microbiota structure between the groups (Figure 4E). At the Class level, compared to the CTRL group,

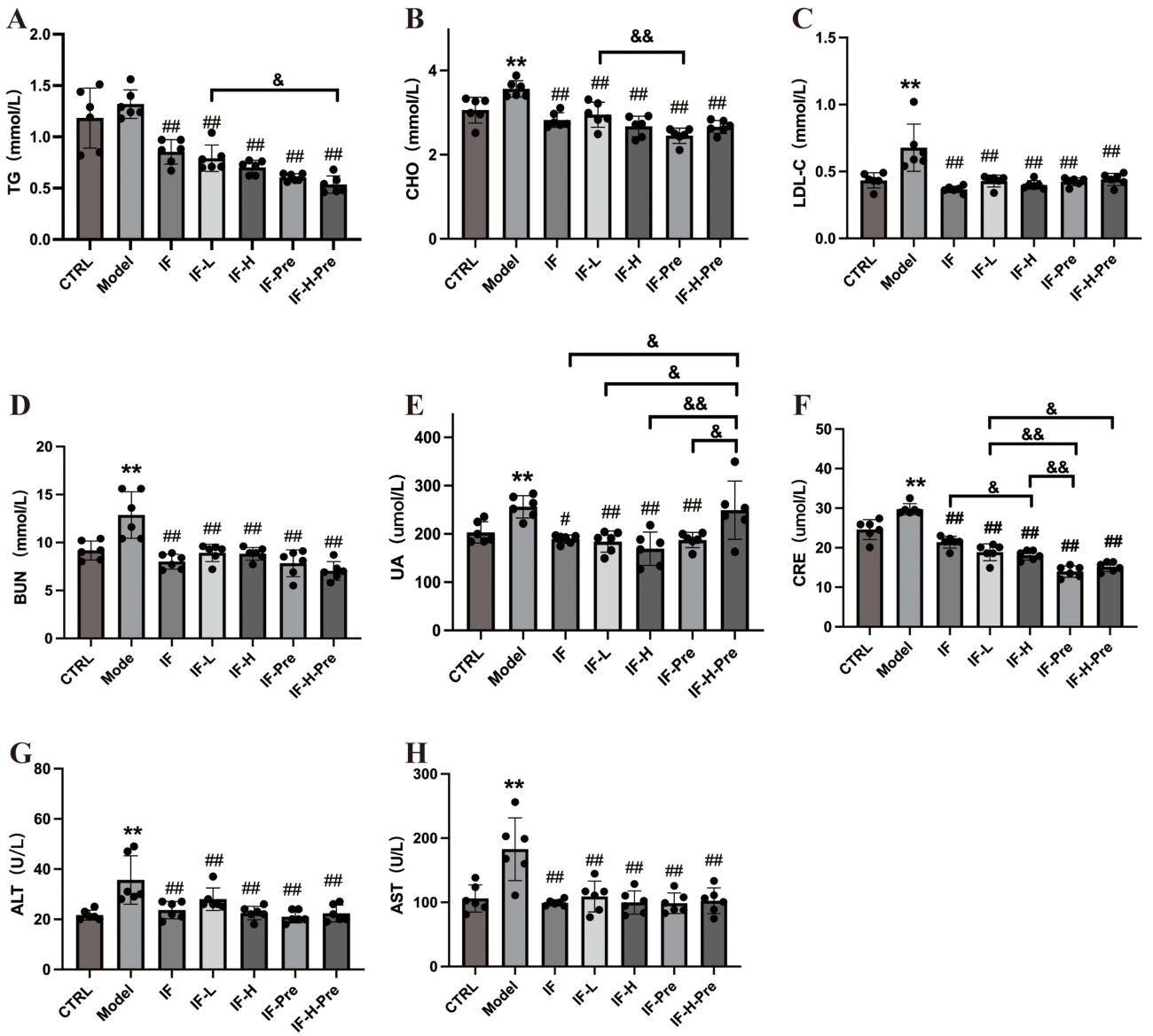

Figure 3 The effects of intermittent fasting combined with Shen-Ling-Bai-Zhu-San and prebiotics on blood biochemical parameters in T2DM mice were investigated. (**A**) Analysis of triglyceride (TG) levels in mice from each group; (**B**) Analysis of cholesterol (CHO) levels in mice from each group; (**C**) Analysis of low-density lipoprotein cholesterol (LDL-C) levels in mice from each group; (**D**) Analysis of blood urea nitrogen (BUN) levels in mice from each group; (**E**) Analysis of uric acid (UA) levels in mice from each group; (**F**) Analysis of creatinine (CRE) levels in mice from each group; (**G**) Analysis of alanine aminotransferase (ALT) levels in mice from each group; (**H**) Analysis of aspartate aminotransferase (AST) levels in mice from each group. $^{**}P<0.01$ vs CTRL group;$^{\#}P < 0.05$ and $^{\#\#}P<0.01$ vs Model group; $^{\&}P<0.05$ and $^{\&\&}P<0.01$ vs different IF groups.

Verrucomicrobiae and *Actinobacteria* decreased in the Model group mice, while the combination of intermittent fasting with prebiotics or with both prebiotics and SLBZS restored the levels of *Verrucomicrobiae* and *Actinobacteria* (Figure 5A). At the Family level, abundance analysis showed significant decreases in *Akkermansiaceae* and *Bifidobacteriaceae* in type 2 diabetic model mice, which were restored after treatment with intermittent fasting combined with prebiotics and SLBZS (Figure 5B–D). *Prevotellaceae* significantly increased in the Model group mice ($P<0.05$), but after intermittent fasting treatment, the content of *Prevotellaceae* in mice significantly decreased ($P<0.01$) (Figure 5E). Similar changes were observed in the heatmap of species abundance at the phylum level (Figure 6A).

The LEfSe results indicated differences in dominant bacterial communities among the six groups of mice gut microbiota (Figure 6B). Specifically, characteristic bacteria in the Model group included *Prevotellaceae,*

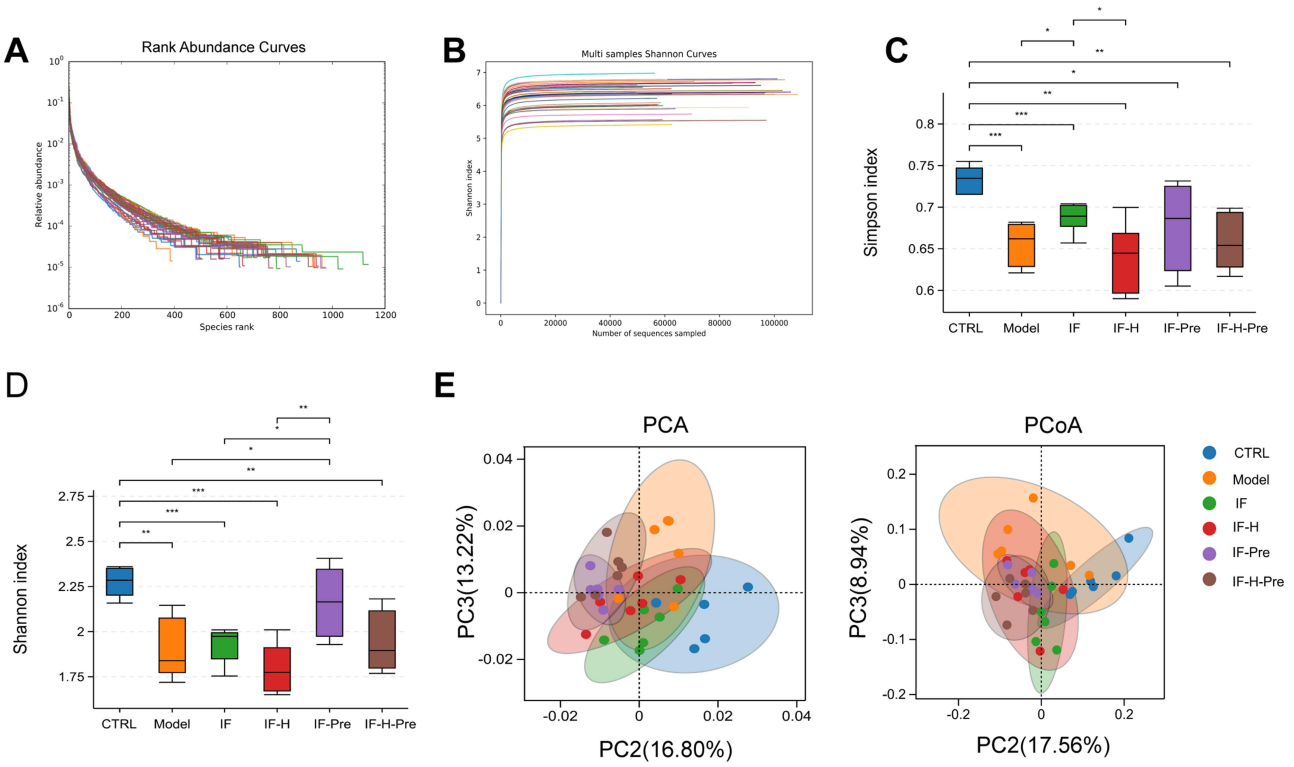

Figure 4 Intermittent fasting combined with SLBZS and prebiotics altered the gut microbiota structure in T2DM mice. CTRL: Control group; Model: Model group; IF: intermittent fasting group; IF-L: IF combined with low concentration of Shen-Ling-Bai-Zhu-San (SLBZS) group; IF-H: IF combined with high concentration of SLBZS group; IF-Pre: IF combined with prebiotics group (IF-Pre); IF-H-Pre: IF combined with high concentration of SLBZS and prebiotics group (IF-H-Pre). (**A**) Rank Abundance Curve. (**B**) Multi-Sample Shannon Curves. (**C**) Shannon Index (n=6). (**D**) Simpson Index (n=6). (**E**) Principal Component Analysis and Principal coordinates analysis (n=6). **P* < 0.05, ***P*<0.01, and ****P*<0.001.

Oscillospirales, and *Ileibacterium*. In contrast, the characteristic bacterial taxa in the IF group included *Firmicutes, Lactobacillales, Ligilactobacillus, Bacilli, Lachnospiraceae, Rikenellaceae* and *Alostipes* (Figure 6C).

The Effects of if Combined with Prebiotics or SLBZS on the Changes of the Gut Microbiota Metabolic Pathways in T2DM Mice

The functional prediction of mouse gut microbiota was conducted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) data analysis method. A comparison between the Model group of type 2 diabetic mice and mice in the pure IF group revealed significant changes in six pathways ($P<0.05$), primarily including global and overview maps, membrane transport, carbohydrate metabolism, energy metabolism, amino acid metabolism, and metabolism of cofactors and vitamins. During the treatment process of mice with IF combined with prebiotics, six pathways underwent changes ($P<0.05$), specifically energy metabolism, membrane transport, signal transduction, global and overview maps, amino acid metabolism, and metabolism of cofactors and vitamins. Additionally, in the comparison between the IF group and IF-H-Pre group mice, there were two pathways exhibited changes ($P<0.05$), including energy metabolism and signal transduction (Figure 7).

The Effects of if Combined with Prebiotics or SLBZS on the Changes of the Expression of Insulin-Related Signaling Pathway Genes in T2DM Mice

To investigate the potential mechanisms underlying the therapeutic effects of IF combined with different doses of SLBZS and prebiotics on type 2 diabetes, we conducted a study on classical insulin signaling transduction pathways related to T2DM, focusing primarily on the PI3K/Akt signaling pathway. In the STZ-HFD-induced T2DM mouse model, the

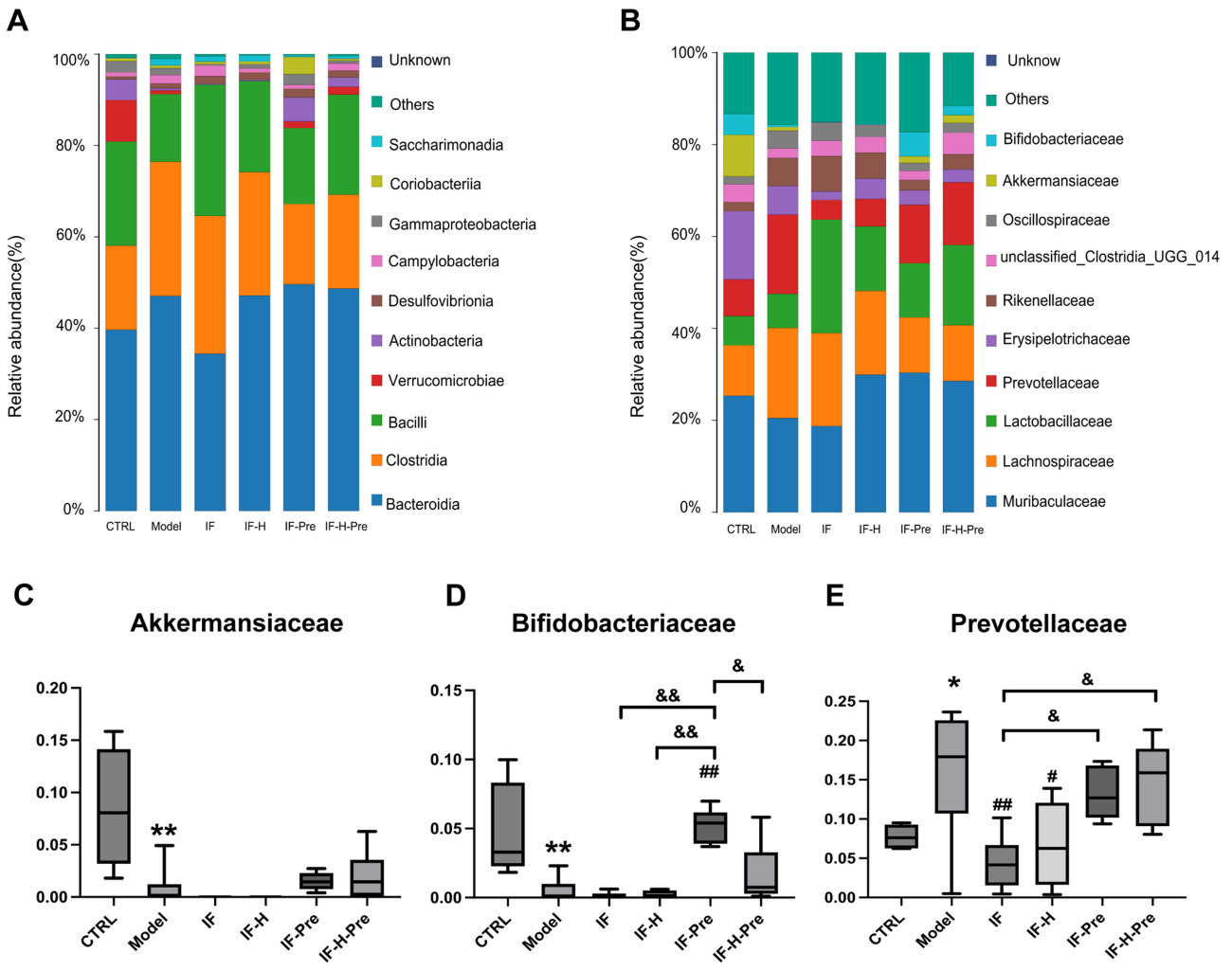

Figure 5 Impact of intermittent fasting combined with SLBZS and prebiotics on the abundance of gut microbiota in T2DM mice. CTRL: Control group; Model: Model group; IF: Intermittent fasting group; IF-L: IF combined with low concentration of Shen-Ling-Bai-Zhu-San (SLBZS) group; IF-H: IF combined with high concentration of SLBZS group; IF-Pre: IF combined with prebiotics group (IF-Pre); IF-H-Pre: IF combined with high concentration of SLBZS and prebiotics group (IF-H-Pre). (**A**) Relative abundance analysis of gut microbiota at the class level (n=6). (**B**) Relative abundance analysis of gut microbiota at the family level (n=6). (**C**) Abundance analysis of *Akkermansiaceae* at the family level in each group of mice (n=6). (**D**) Abundance analysis of *Bifidobacteriaceae* at the family level in each group of mice (n=6). (**E**) Abundance analysis of *Prevotellaceae* at the family level in each group of mice (n=6). **P* < 0.05 and ***P*<0.01 vs CTRL group;$^{\#}$*P* < 0.05 and $^{\#\#}$*P*<0.01 vs Model group; $^{\&}$*P*<0.05,$^{\&\&}$ *P*<0.01 vs different IF groups.

mRNA expression levels of key factors in the insulin signaling pathway varied to different degrees. Compared to the normal group, the mRNA expression levels of insulin receptor substrate (*IRS1*), insulin receptor (*INSR*), and glucose transporter 2 (*GLUT2*) were significantly downregulated in the T2DM model group ($P<0.05$), while the mRNA expression level of glycogen synthase kinase-3β (*GSK-3β*) was significantly upregulated ($P<0.05$). Treatment with IF led to a partial upregulation of the relative expression levels of *INSR, IRS1*, and *GLUT2* to varying degrees and a reduction in the expression level of *GSK-3β*. Furthermore, treatment with prebiotics alone or in combination with high-dose of SLBZS significantly upregulated the relative expression levels of *INSR* and *IRS1* genes compared to IF alone ($P<0.01$), suggesting that the use of prebiotics can significantly upregulate the gene expression of *INSR* and IRS1 (Figure 8).

Discussion

In this study, the STZ-HFD induced type 2 diabetic mice exhibited pathological changes and symptoms similar to those of type 2 diabetic patients, including hyperglycemia, increased body weight, impaired glucose tolerance, and pancreatic islet dysfunction, indicating the successful preparation of the animal model. It is noteworthy that the Model group mice

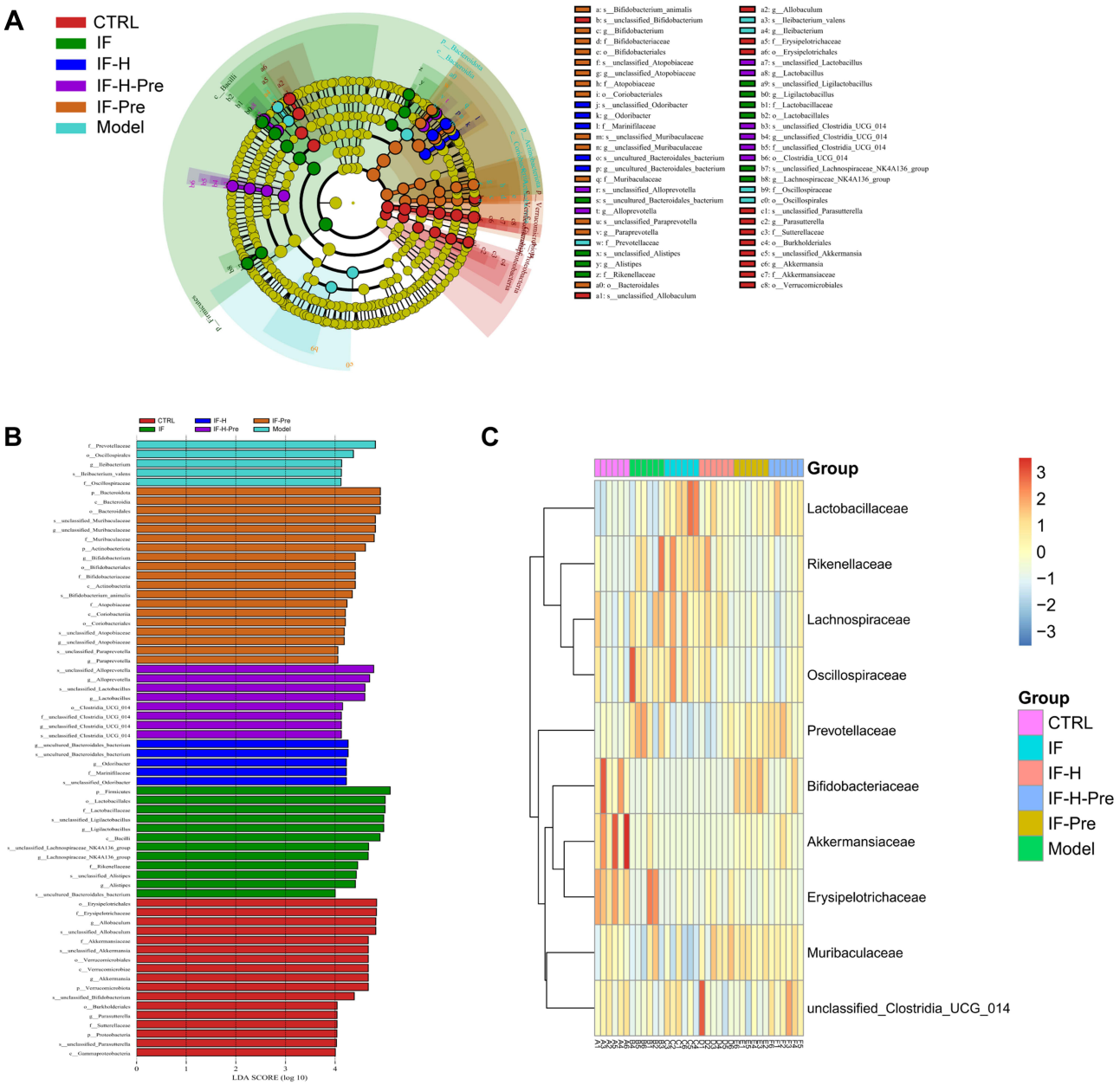

Figure 6 Differential analysis among groups and heatmap analysis of the impact of intermittent fasting combined with SLBZS and prebiotics on the abundance of gut microbiota in T2DM mice. CTRL: Control group; Model: Model group; IF: Intermittent fasting group; IF-L: IF combined with low concentration of Shen-Ling-Bai-Zhu-San (SLBZS) group; IF-H: IF combined with high concentration of SLBZS group; IF-Pre: IF combined with prebiotics group (IF-Pre); IF-H-Pre: IF combined with high concentration of SLBZS and prebiotics group (IF-H-Pre). (**A**) LEfSe analysis cladogram diagram. In the figure, circles from center to outward layers represent taxonomic level from phylum to species. The node on circles represents a term on corresponding taxonomic level. The size of the dots indicates relative abundance. Colouring: Species with no significant difference are coloured in yellow. Otherwise, the nodes were coloured according to the group with the highest relative abundance, which helps visualize the relevance of different biological aspects (n=6). (**B**) Linear discriminant analysis (LDA) analysis. LDA < 4. Y-axis: Features that shown significant difference between groups; X-axis: Log10 of LDA score. The features were sorted according to LDA score. A longer bar indicates a more significant difference. The bars were coloured according to the group with highest abundance of corresponding feature (n=6). (**C**) Result of species abundance clustering heatmap at the class level.

exhibited the lowest serum insulin levels. As type 2 diabetes progresses, insulin fails to function properly, leading to continuously rising blood glucose levels. This stimulates the body to release more insulin to regulate the blood sugar, but over time, pancreatic β-cell function is impaired, resulting in a gradual reduction in insulin secretion, further elevating blood glucose levels.[33] This may account for the higher blood glucose levels but reduced insulin content observed in the Model group mice in this experiment. Furthermore, in the T2DM mice of this study, levels of CHO and LDL-C were significantly higher than those in the CTRL group, indicating a certain degree of lipid metabolism abnormality in the

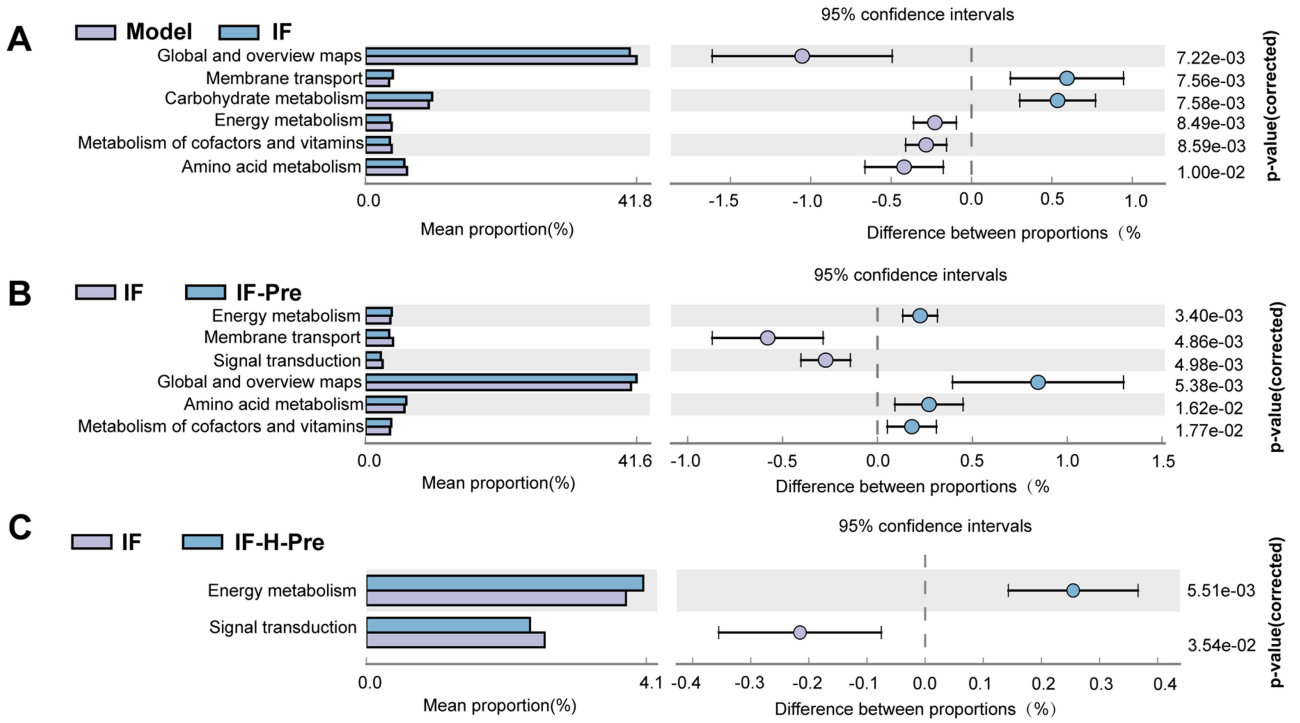

Figure 7 Differential analysis of gut microbial metabolic pathways in various groups treated with intermittent fasting combined with SLBZS and prebiotics using PICRUSt2. CTRL: Control group; Model: Model group; IF: Intermittent fasting group; IF-L: IF combined with low concentration of Shen-Ling-Bai-Zhu-San (SLBZS) group; IF-H: IF combined with high concentration of SLBZS group; IF-Pre: IF combined with prebiotics group (IF-Pre); IF-H-Pre: IF combined with high concentration of SLBZS and prebiotics group (IF-H-Pre). (**A**) Differential analysis of gut microbial functions between the T2DM mice model group and the IF group (n=6). (**B**) Differential analysis of gut microbial functions between the IF group and the IF-Pre group (n=6). (**C**) Differential analysis of gut microbial functions between the IF group and the IF-H-Pre group (n=6).

T2DM mice. The biochemical indicators such as ALT, AST for liver function, and BUN, UA, and CRE for kidney function were analyzed. The results showed abnormalities in liver and kidney function in the T2DM mice induced by STZ-HFD in this experiment.

It was found that IF not only significantly improved the abnormal indicators of STZ-HFD-induced type 2 diabetic mice but also restored the abnormalities in biochemical indicators such as blood lipids, liver function, and kidney function in T2DM mice. Furthermore, the use of prebiotics or different concentrations of SLBZS had varying effects on type 2 diabetic mice. Analysis of the major components of SLBZS showed that it contains 5.97% polysaccharides, 4.93% proteins, 0.15% polyphenols, 0.027% flavonoids, and 0.078% saponins The high performance liquid chromatography analysis on SLBZS from other researchers indicated that the five principal chemical constituents of SLBZS were liquiritin, lobetyolin, glycyrrhizic acid ammonium salt, platycodin and atractylenolide III[34] Compared to low concentration of SLBZS, the high concentration of SLBZS showed better efficacy in treating type 2 diabetes with combination of IF. Additionally, under conditions of gut microbiota dysbiosis induced by STZ-HFD, IF combined with prebiotics exhibited better therapeutic effects.

Diabetes is closely associated with the gut microbiota, as evidenced by the corresponding information in this study. Diversity and abundance are known as important indicators for assessing the health status of gut microbiota. Compared to the CTRL group, 16S rRNA sequencing analysis indicated a significant decrease in the Shannon index and Simpson index in STZ-HFD-induced type 2 diabetic mice, suggesting a notable reduction in gut microbiota alpha diversity in these animals. Following IF treatment, the Simpson index of mice in the Model group significantly increased, indicating that IF can partially restore gut microbiota diversity in type 2 diabetic mice. Additionally, the use of prebiotics significantly increased the Shannon index in type 2 diabetic mice. Through principal component analysis of gut microbiota structure, it was found that the gut microbiota structure of the IF-Pre group and IF-H-Pre group mice was closer to that of the CTRL group mice.

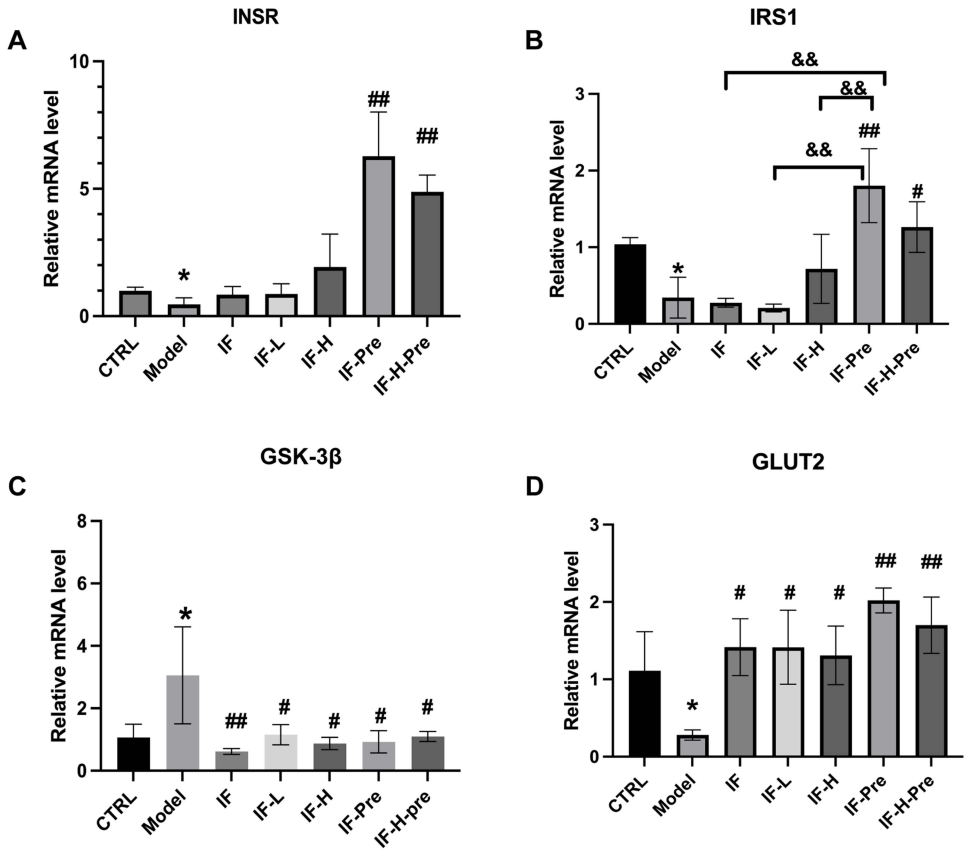

Figure 8 The effect of intermittent fasting combined with SLBZS and prebiotics on the relative expression levels of key genes in the insulin signaling pathway in each group of mice. CTRL: Control group; Model: Model group; IF: Intermittent fasting group; IF-L: IF combined with low concentration of Shen-Ling-Bai-Zhu-San (SLBZS) group; IF-H: IF combined with high concentration of SLBZS group; IF-Pre: IF combined with prebiotics group (IF-Pre); IF-H-Pre: IF combined with high concentration of SLBZS and prebiotics group (IF-H-Pre). (**A**) Relative expression level of the *INSR* gene. (**B**) Relative expression level of the *IRS1* gene. (**C**) Relative expression level of the *GSK-3β* gene. (**D**) Relative expression level of the *GLUT2* gene. $^{*}P < 0.05$ vs CTRL group; $^{\#}P < 0.05$ and $^{\#\#}P<0.01$ vs Model group; $^{\&\&}P<0.01$ vs different IF groups.

Further analysis revealed that at the class level, *Verrucomicrobiae* and *Actinobacteria* were significantly reduced in the gut microbiota of model mice. At the family level, *Akkermansiaceae* and *Bifidobacteriaceae* were also significantly decreased. *Akkermansiaceae* is a family within the phylum *Verrucomicrobia*. Numerous studies have shown that the abundance of *Akkermansiaceae* was reduced in the gut microbiota of metabolic disease model mice compared to healthy mice, and supplementation of *Akkermansiaceae* effectively improves related symptoms in diseased mice.[35] Gastric gavage of *Akkermansiaceae* significantly improves intestinal mucosal barrier dysfunction and metabolic disorders in high-fat diet mice.[36] *Actinobacteria* is a common group of short-chain fatty acid-producing bacteria. Increased secretion of short-chain fatty acids (SCFAs) promotes elevated levels of GLP-1 in the body,[37] enabling GLP-1 to exert multiple effects involved in regulating blood glucose homeostasis and organism metabolism.[38,39] SCFAs can activate FFAR3 to stimulate enteroendocrine L cells, resulting in the slow release of the intestinal hormone peptide YY (PYY, an enteroendocrine hormone that reduces gut motility), which inhibits gastric acid secretion, gastric emptying, and food transit time, thereby controlling appetite, increasing satiety, reducing food intake in type 2 diabetic patients, and lowering blood glucose levels.[40] Several studies have found a decrease of *Bifidobacteriaceae* in patients with T2DM.[41–44] Additionally, antidiabetic drugs such as metformin,[45,46] acarbose,[47,48] and herbal[49] preparations can increase the abundance of *Bifidobacterium* while reducing blood sugar levels. *Bifidobacteriaceae*, as prebiotics, have been shown to reduce fat accumulation and promote host nutrient absorption in both humans and animals.[50] Among them, *Bifidobacterium* adolescentis Z25 has demonstrated the ability to alleviate metabolic syndrome, including disturbances in glucose and lipid metabolism, tissue damage, and gut microbiota dysbiosis.[51] Research has confirmed that oral supplementation of *Bifidobacterium* can alleviate insulin resistance and improve glucose tolerance in obese mice, as well

as inhibit fat accumulation.[52] In this study, IF did not significantly increase the abundance of *Akkermansiaceae* and *Bifidobacteriaceae*, but the combination of prebiotics and high concentration of SLBZS significantly increased the abundance of these two bacterial groups in the gut of type 2 diabetic mice. Compared to the combination of prebiotics and high concentration of SLBZS, IF combined with prebiotics showed better efficacy in increasing the abundance of *Akkermansiaceae* and *Bifidobacteriaceae*. Additionally, PICRUSt2 analysis demonstrated that IF enhances pathways such as membrane metabolism and carbohydrate metabolism in type 2 diabetic mice. Blood glucose concentration is an important indicator reflecting the status of glucose metabolism in the body. The enhancement of carbohydrate metabolism promotes glucose absorption, glycogen synthesis, and reduces blood glucose concentration. On the basis of IF, the combination of prebiotics also enhances pathways such as energy metabolism,[53] amino acid metabolism, and metabolism of cofactors and vitamins. This provides a reference basis for the use of prebiotics to improve the efficiency of IF in daily life.

In this study, we found that IF can treat symptoms associated with STZ-HFD-induced type 2 diabetic mice and improve the reduced diversity of their gut microbiota, providing new insights into the treatment of type 2 diabetes. Based on this, it was shown that the combined use of different doses of SLBZS and prebiotics has similar therapeutic effects. Prebiotics are substances that cannot be broken down, absorbed, or utilized by the human body. Instead, they are broken down and utilized by the gut microbiota in the colon, promoting the growth of beneficial bacteria, improving gut microbiota balance, and enhancing diversity, thereby improving gut microbiota imbalance.[54] IF can adjust dietary habits to improve blood glucose levels in diabetic mice. The combined use of these two methods can partially replace traditional drug therapy, reducing the medication burden on diabetes patients. However, in improving gut microbiota imbalance caused by type 2 diabetes, IF combined with prebiotics has better effects compared to other combined interventions. It is worth noting that SLBZS, as a type of traditional Chinese medicine, acts as a tonic agent in traditional Chinese medicine theory, with the effect of tonifying qi and invigorating the spleen. However, IF requires patients to adhere to it for a long time and may bring about certain lifestyle changes, such as hunger caused by prolonged fasting periods. Therefore, for some patients, IF may not be an easily acceptable treatment method. In this context, SLBZS can not only be used in conjunction with IF to lower blood glucose levels in diabetic mice but also alleviate discomfort to some extent caused by IF.

Numerous studies have found that the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway and its downstream PI3K/Akt insulin signaling pathway are associated with the pathogenesis of insulin resistance (IR) in the liver of patients with T2DM.[55,56] Insulin receptor substrates (IRS) primarily exist in insulin-sensitive tissues and are key signaling proteins associated with insulin and its function.[57,58] The initial step in the insulin signaling transduction pathway is the binding of insulin to the insulin receptor, which then activates IRS. GSK-3β is a key enzyme downstream of PI3K/Akt involved in glycogen synthesis, widely expressed in animal tissues and cells.[59] Akt can inhibit the activity of GSK-3β, increasing glycogen synthesis and thereby reducing blood glucose levels. Additionally, studies have shown that GSK-3β inhibitors can promote the proliferation of INS-1 cells, leading to a decrease in blood glucose levels. GLUT2 is a major liver transport protein.[60] Under normal physiological conditions, GLUT2 can promptly respond to changes in blood glucose concentration, promoting cellular uptake and utilization of glucose. In the liver tissues of STZ-HFD-induced type 2 diabetic mice, the mRNA relative expression levels of key factors in the insulin signaling pathway exhibited varying degrees of changes. Results indicated that compared to the normal group, the mRNA expression levels of *IRS1, INSR*, and *GLUT2* were significantly down-regulated, while the expression level of *GSK-3β* mRNA was significantly upregulated in the Model group, suggesting the occurrence of IR and metabolic disorders in glucose and lipids in the liver tissues of T2DM mice. After intermittent fasting treatment, the mRNA relative expression levels of *INSR* and *GLUT2* were upregulated to varying degrees, while *GSK-3β* mRNA expression was significantly downregulated. Importantly, the mRNA relative expression levels of *INSR* and *IRS1* in the IF-Pre and IF-H-Pre groups of mice were significantly upregulated compared to CTRL group. Upregulation of the *INSR* gene expression leads to an increase in insulin receptor numbers, promoting the binding of *INSR* to *IRS1* and increasing insulin sensitivity. Upon activation, *IRS1* further activates *PI3K*, promoting Akt phosphorylation, thereby regulating glucose and lipid metabolism. Activated *Akt* increases the phosphorylation level of *GSK-3β*, inhibiting its activity, and promoting glycogen synthesis in the liver. Furthermore, translocation of GLUT2

from the cytoplasm to the cell membrane enhances hepatic glucose transport, promoting glucose uptake and utilization, thereby maintaining glucose homeostasis.

Conclusion

In summary, our study demonstrates that the combined use of prebiotics and SLBZS on the basis of intermittent fasting can effectively treat type 2 diabetes mellitus and ameliorate its associated symptoms, providing a novel therapeutic strategy. In future research, we will further investigate the application value of this combined intervention in the treatment of other diseases, with the goal of offering additional options for clinical therapy.

Data Sharing Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

We confirm that we have contributed to and reviewed the content of this manuscript, and we agree to its submission. We affirm that all authors listed have agreed to be so listed and have seen and approved the submitted version of the manuscript.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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