

Screening of Potential Probiotic *Lactobacillaceae* and Their Improvement of Type 2 Diabetes Mellitus by Promoting PI3K/AKT Signaling Pathway in db/db Mice

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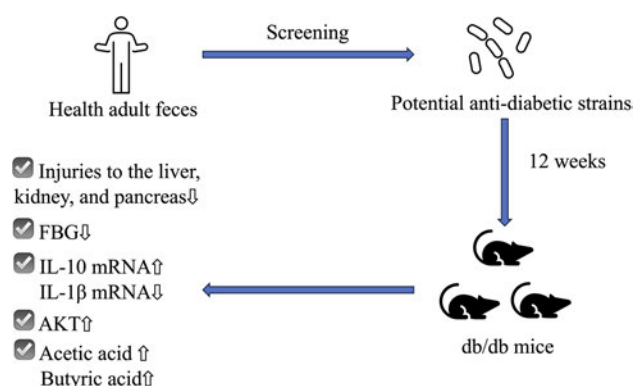
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Abstract

The study aimed to isolate *Lactobacillaceae* strains with *in vitro* hypoglycemic activity and probiotic properties and to determine their antidiabetic abilities *in vivo*. *Lactiplantibacillus plantarum* 22, *L. plantarum* 25, *Limosilactobacillus fermentum* 11, and *L. fermentum* 305 with high *in vitro* hypoglycemic activity were screened from 23 strains of *Lactobacillaceae* isolated from human feces and identified by 16S rDNA sequencing. The fasting blood glucose (FBG) of the mice was recorded weekly. After 12 weeks, liver, kidney, and pancreas tissues were stained with hematoxylin and eosin (H&E) to observe histomorphology; the inflammatory factors were assayed by Quantitative Real-time PCR; PI3K and AKT were measured by Western blot; the short-chain fatty acids (SCFAs) were determined by LC-MS/MS. Inhibitory activities of *L. plantarum* 22, *L. plantarum* 25, *L. fermentum* 11, and *L. fermentum* 305 against α -amylase were $62.29 \pm 0.44\%$, $51.81 \pm 3.65\%$, $58.40 \pm 1.68\%$, and $57.48 \pm 5.04\%$, respectively. Their inhibitory activities to α -glucosidase were $14.89 \pm 0.38\%$, $15.32 \pm 0.89\%$, $52.63 \pm 3.07\%$, and $51.79 \pm 1.13\%$, respectively. Their survival rate after simulated gastrointestinal test were $12.42 \pm 2.84\%$, $9.10 \pm 1.12\%$, $5.86 \pm 0.52\%$, and $8.82 \pm 2.50\%$ and their adhesion rates to Caco-2 cell were $6.09 \pm 0.39\%$, $6.37 \pm 0.28\%$, $6.94 \pm 0.27\%$, and $6.91 \pm 0.11\%$, respectively. The orthogonal tests of bacterial powders of the four strains



showed that the maximum inhibitory activities to α -amylase and α -glucosidase were $93.18 \pm 1.19\%$ and $75.33 \pm 2.89\%$, respectively. The results showed that the mixture of *Lactobacillaceae* could lower FBG, reduce inflammation, and liver, kidney, and pancreas damage, promote PI3K/AKT signaling pathway, and increase the content of SCFAs. The combination of *L. plantarum* 22, *L. plantarum* 25, *L. fermentum* 11, and *L. fermentum* 305 can potentially improve type 2 diabetes mellitus (T2DM).

Key words: *Lactobacillaceae*, type 2 diabetes mellitus, PI3K/AKT pathway, probiotics properties, short chain fatty acids

Introduction

With the increasing number of diabetic patients, diabetes, especially type 2 diabetes mellitus (T2DM), is becoming an increasingly prevalent health issue in the

21st century (Cao et al. 2020; Wang et al. 2021a). Hyperglycemia is the main symptom of T2DM, primarily caused by insulin resistance or inadequate insulin production (Wu et al. 2021). The treatment of T2DM mainly includes two standard methods. The one is insulin

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injections, which could result in insulin resistance. Another method is orally administered hypoglycemic medications, which may cause adverse reactions and subsequent failures (Sun et al. 2020). It necessitates the development of non-toxic treatment options with hypoglycemic effects without side effects.

Many drugs used to treat diabetes, acarbose and miglitol are among the most commonly used. Unfortunately, these drugs have some adverse effects, including diarrhea, flatulence, and abdominal pain (Rosas-Ramírez et al. 2018). Probiotics have a promising future in treating T2DM in humans, so it is important to develop alternatives to current antidiabetic medicine (Awad et al. 2018). It has been demonstrated that probiotics, which are generally considered to be ingested microorganisms that have favorable effects on the host, are effective in preventing and alleviating T2DM through different mechanisms, such as lowering blood glucose or inhibiting pro-inflammatory markers (Hill et al. 2014; Sharma and Shukla 2020; Sun et al. 2020; Zendeboodi et al. 2020; Zepeda-Hernández et al. 2021). *Limosilactobacillus fermentum* 21828 showed probiotic and hypoglycemic activities *in vitro* (Wang et al. 2021b). *Ligilactobacillus salivarius* AP-32 and *Limosilactobacillus reuteri* GL-104 improved T2DM by reducing fasting blood glucose (FBG) levels and attenuating liver and kidney damage (Hsieh et al. 2020). *L. fermentum* MCC2759 and MCC2760 have shown positive results in a T2DM model by modifying lipid and glucose profiles, reducing inflammatory factors in the liver, and strengthening intestinal barrier function (Archer et al. 2021). After *Lactiplantibacillus plantarum* LRCC5314 intervened in diabetic model mice, the expressions of glucose transport-related genes Glut4 and adiponectin were increased, and the expressions of TNF- α and IL-6 decreased (Youn et al. 2021). It was reported in both *in vitro* and *in vivo* studies that *L. plantarum* YJ7 showed antihyperglycemic potential (Zhong et al. 2021). The effects of probiotics from different sources are not identical. Vemuri et al. (2018) found that compared with plant-derived *L. plantarum* UALp-05 and dairy-derived *Streptococcus salivarius* subsp. *thermophilus* UAS-09, only human-derived probiotics *Limosilactobacillus acidophilus* DDS-1 and *Bifidobacterium animalis* ssp. *lactis* UABla-12 could induce human colon cancer cells to secrete IL-10. Therefore, probiotics may be used as a non-toxic treatment with hypoglycemic effects and no side effects, and human-derived probiotics may be more suitable.

By inhibiting the action of α -amylase and α -glucosidase, carbohydrates are not broken down and absorbed as quickly, reducing the glucose rate that enters the bloodstream after a meal (Barros et al. 2021; Zhang et al. 2022). It helps reduce post-meal spikes in blood glucose levels, which benefits people with diabetes. Therefore,

inhibiting the action of α -amylase and α -glucosidase is critical to treating T2DM (Gong et al. 2020). The probiotics that may improve T2DM were screened by examining the inhibitory activities of α -amylase and α -glucosidase. The effects of multiple probiotic strains are superior to those of a single strain in terms of gut function, gut microbiota regulation, intestinal inflammation, depression, and neurological disorders (Chapman et al. 2011; Li et al. 2019; Dehghani et al. 2022).

The 16S rDNA sequencing was used to confirm the identification of the selected strain (Jomehzadeh et al. 2020; Lin et al. 2020). Therefore, four novel strains of *Lactobacillaceae* with high inhibitory activity against α -glucosidase and α -amylase were isolated from the feces of healthy adults and identified by the 16S rDNA sequencing in this study. This study is the first that investigates the hypoglycemic ability of the *Lactobacillaceae* mixture with good probiotic properties from the feces of healthy adults. A theoretical foundation is provided for applying human-derived lactic acid bacteria to T2DM research in the future.

Experimental

Materials and Methods

Isolation of human *Lactobacillaceae*. The use of fecal samples is approved by a healthy fecal provider who has not taken any medication in the past year and has undergone a professional physical examination at the Chinese People's Liberation Army (PLA) General Hospital (Beijing, China). Fecal samples (10 g) from healthy adults were resuspended in 100 ml de Man, Rogosa and Sharp medium (MRS; Beijing Land Bridge Technology Co., Ltd., China) and stationary cultivated for 24 h at 37°C. Then, cultures were diluted and spread onto the MRS agar plates. After cultivation for 24 h at 37°C, colonies were picked up and added to 10 ml MRS medium. After cultivation for 12 h at 37°C, the culture broth was added to 10 ml MRS medium with 5% (v/v) inoculation volume. Finally, a diluted suspension of the culture broth was plated onto MRS agar plates, and colonies were counted.

Identification of isolates. Total DNA was extracted from bacterial cultures in MRS broth. 200 μ l of cultures was collected in 1.5 ml tube and centrifuged at 9,000 $\times$ g for 2 min. After the cells were washed, excess liquid was removed, and 20 μ l bacterial lysate (NaOH/SDS) was added, then incubated at room temperature for 20 min and diluted 20 times. Finally, 2 μ l of supernatant was centrifuged for 2 min. The amplification procedure was pre-denaturation (94°C, 5 min), 40 cycles of denaturation (94°C, 1 min), annealing (55°C, 60 s), extension (72°C, 60 s), and a final extension at 72°C for 10 min.

DNA templates were amplified by PCR using the universal primers amplifying a region of the 16S rRNA gene: 27F (5'-AGAGTTTGTATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Kunduhoglu and Hacıoglu 2021). After purification of the PCR product from the agarose gel, the samples were sent for sequencing. The resulting sequences were compared with the online tool BLAST.

α -Amylase inhibitory activity assay. Based on Barapatre et al. (2015) with some variations, the α -amylase inhibitory activity was measured. 10 μ l of 0.02 M sodium phosphate buffer (pH 6.9) containing α -amylase (0.5 mg/ml) and 50 μ l bacterial suspension (10^9 CFU/ml) were mixed and incubated at 37°C for 10 min. Then, 50 μ l of 1% (w/v) starch dissolved in 0.02 M sodium phosphate buffer (pH 6.9) was added and further incubated at 37°C for 10 min. The reaction was terminated by adding 20 μ l of DNS reagent. Then, the reaction mixture was centrifuged at $8,000 \times g$ at 4°C for 5 min. The α -amylase activity was detected at 540 nm, and inhibitory activity was calculated by using the formula below:

$$\text{Inhibition (\%)} = [1 - ((\gamma_1 - \delta_1) / (\alpha_1 - \beta_1))] \times 100\% \quad (1)$$

where α_1 – the absorbance with α -amylase and without the sample, β_1 – the absorbance without α -amylase or the sample, γ_1 – the absorbance with α -amylase and the sample, and δ_1 – the absorbance with the sample but without α -amylase.

α -Glucosidase inhibitory activity assay. The α -glucosidase inhibitory activity was determined by the method of Nguyen et al. (2018). The mixture included 50 μ l of 0.1 M phosphate buffer (pH 6.9), 20 μ l of bacterial suspension (10^9 CFU/ml), and 20 μ l of p-nitrophenyl- α -D-glucopyranoside (PNPG, 5 mM). After the mixture was incubated for 10 min at 37°C, 10 μ l of 0.5 U/ml α -glucosidase solution was added. The mixture was incubated for 10 min at 37°C; then the reaction was stopped by adding 100 μ l of sodium carbonate (0.02 M). The reaction mixture was centrifuged at $8,000 \times g$ for 5 min (4°C). Then, the supernatant was diluted with 1 ml distilled water, and the absorbance was measured at 405 nm. The inhibitory activity was calculated by using the formula below:

$$\text{Inhibition (\%)} = [1 - ((\gamma_2 - \delta_2) / (\alpha_2 - \beta_2))] \times 100\% \quad (2)$$

where α_2 – the absorbance with α -glucosidase and without the sample, β_2 – the absorbance without α -glucosidase or the sample, γ_2 – the absorbance with α -glucosidase and the sample, and δ_2 – the absorbance with the sample but without α -glucosidase.

Resistance to gastrointestinal juice. Strain survivability was assessed using an *in vitro* digestion model. Artificial saliva, gastric juice, and intestinal juice were

manufactured as described by Zhu et al. (2015). Following washing, bacteria (1 ml, 1.0×10^9 CFU/ml) were simulated digestion at 37°C with peristaltic contraction, including a) suspension in 5 ml artificial saliva for 5 min; b) resuspension in 10 ml artificial gastric juice, incubation for 2 h; c) resuspension in 10 ml artificial intestinal juice, incubation for 2 h. Then, a diluted suspension of simulated digested bacteria was plated onto MRS agar plates and cultivated for 24 h at 37°C. The colonies were counted:

$$\text{Survival rate (\%)} = \frac{M_1}{M_0} \times 100\% \quad (3)$$

where M_1 – the number of viable bacteria after the process, M_0 – the number of viable bacteria before the process.

Adhesion to Caco-2 cell. Bacterial adhesion to Caco-2 cell cultures was investigated according to Fonseca et al. (2021). After cultivation in MRS broth for 24 h at 37°C and washing twice with phosphate-buffered solution, the bacteria were resuspended in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich Co., Ltd., USA) at a density of 10^9 CFU/ml. Caco-2 cells were then cultivated for 1 h at 37°C in an atmosphere of 5% CO_2 with a 1 ml culture suspension of bacteria. After being washed three-time with PBS, treated with 1 ml Triton-X solution, and cultivated for 5 min at 37°C, the culture medium was serially diluted and plated onto MRS agar to count the bacterial colonies:

$$\text{Adhesion ability (\%)} = \frac{N_1}{N_0} \times 100\% \quad (4)$$

where N_1 – the number of viable bacteria after the process, N_0 – the number of viable bacteria before the process.

Preparation and counting of *Lactobacillaceae* powder. After incubating at 37°C for 24 h, four *Lactobacillaceae* strains were centrifuged for 5 min at 4°C and twice washed with sterile water to extract the bacterial cells. Then, the bacteria were freeze-dried to produce powders. Four *Lactobacillaceae* powders were suspended in sterile water, respectively. A diluted powder suspension was plated onto MRS agar plates to encounter the number of bacterial colonies.

Inhibitory activities to α -glucosidase and α -amylase of *Lactobacillaceae* powders. An L_9 (3^4) orthogonal table was designed. The inhibitory activities to α -amylase and α -glucosidase were used as the inspection index. *L. fermentum* 11 (A), *L. fermentum* 305 (B), *L. plantarum* 22 (C), and *L. plantarum* 25 (D) were used as the experimental factors. Each factor was designed with three levels, and the factors and levels of orthogonal tests for the inhibitory activities to α -amylase and α -glucosidase were shown in Table I.

Animal experiment. All animals were maintained in a specific pathogen-free (SPF) environment. Under the 12-hour light cycle, the animals were permitted

Table I
Factors and levels of the orthogonal experiment
of *Lactobacillaceae* powders.

Level	A (<i>Limosi- lactobacillus fermentum</i> 11 (CFU/ml))	B (<i>Limosi- lactobacillus fermentum</i> 305 (CFU/ml))	C (<i>Lactiplanti- bacillus plantarum</i> 22 (CFU/ml))	D (<i>Lactiplanti- bacillus plantarum</i> 25 (CFU/ml))
1	10 ⁸	10 ⁸	10 ⁸	10 ⁸
2	10 ⁹	10 ⁹	10 ⁹	10 ⁹
3	10 ¹⁰	10 ¹⁰	10 ¹⁰	10 ¹⁰

to access food and water at any time. In the following experiment, mice were divided into three groups after an adjustment period of 7 days: the control group (N group, 12 wt/wt mice), the diabetic model group (M group, 12 db/db mice), and the probiotic-treated group (ML group, 12 db/db mice with the *Lactobacillaceae* mixture intervention). The freeze-dried bacterial powders of four strains (*L. plantarum* 22, *L. plantarum* 25, *L. fermentum* 11, and *L. fermentum* 305) were mixed (the number of viable bacteria was 1.0 × 10⁹ CFU/ml for each strain). A dose of 10 ml/kg body weight was administered intragastrically to the ML group. The body weights (BW) and the FBG level of mice were recorded every week. A standard glucometer was used to determine the FBG level in the blood from the tail vein (F. Hoffmann-La Roche Co., Ltd., Switzerland).

Histopathological analysis. The liver, kidney, and pancreas tissues were fixed in 4% (w/v) paraformaldehyde for 24 h, embedded in paraffin, and sectioned (5 μm). Sections were observed using an optical microscope (Motic China Group Co., Ltd., China). The sections were stained with hematoxylin and eosin (H&E), according to Zhao et al. (2021).

Quantitative Real-time PCR. The colon tissues (100 mg) were homogenized in pre-cold RNA extraction solutions (1 ml), and centrifuged at 12,000 × g at 4°C for 10 min to obtain the total RNA. Reverse transcription was performed using the PrimeScript RT kit (Promega Corporation, USA), and the conditions were 42°C for 60 min then 70°C for 5 min. cDNA was amplified using SYBR Green Real-Time PCR Master Mix Kit (Servicebio Co., Ltd., China).. The amplification procedure was as follows: pre-denaturation (95°C, 10 min), denaturation (95°C, 15 s), and annealing/extension (60°C, 60 s), the dissolution curve (60–95°C, the temperature is increased by 0.3°C every 15 s). Relative fold changes were performed using the 2^{-ΔΔCt} method. The internal reference was GAPDH, and the primers used for qRT-PCR are shown below (Table II).

Western blot. The liver tissue (100 mg) was homogenized in pre-chilled RIPA lysis buffer solution (1 ml), and centrifuged at 12,000 × g and 4°C for 10 min to

extract total cell lysate. The protein concentration of cell lysate was measured using the BCA protein concentration assay kit (Servicebio Co., Ltd., China). Proteins were separated using 10% SDS-PAGE, transferred to nitrocellulose (PVDF) membranes, then blocked with 5% skim milk for 1 h at room temperature on a shaker. PVDF membranes were incubated with primary antibodies against PI3K and AKT at a 1:1000 dilution overnight at 4°C, followed by incubation with secondary antibodies for 30 min at room temperature. Finally, protein bands were visualized by enhanced ECL reagents, using β-actin as an internal control, and quantification of the bands was performed using the Image J program.

UPLC-MS/MS analysis. The cecal contents (50 mg) were vortexed in methanol/water solution (1:1 v/v, 1 ml) and centrifuged at 12,000 × g and 4°C for 5 min to collect the supernatant. Then the supernatant (50 μl) was derivatized in 50 μl derivatizing reagent and analyzed with UPLC-MS/MS. UPLC-MS/MS analysis was performed using an Ultra Performance Liquid Chromatography I-Class (Waters Co., Ltd., USA) coupled with a Tandem Mass Spectrometry (Waters Co., Ltd., USA). A 13-min linear gradient flow rate of 0.3 ml/min was used to inject samples into a UPLC RP Column (1.6 μm, 2.1 mm × 100 mm). Eluents A (0.1% formic acid in water) and B (methanol: isopropyl alcohol is 4: 1) were used for the system. As follows, the solvent gradient: 5% B, 0 min; 5–15% B, 2.0 min; 15–55% B, 9.0 min; 55–100% B, 11.0 min; 100–5% B, 11.1 min; 5% B, 13 min. The Ultra Performance Liquid Chromatography operated with 150°C source temperature, 2.0 kV capillary voltage, 30.0 V cone voltage, 450°C desolvation temperature, 150 l/h cone gas flow, 1000 l/h desolvation gas flow, 0.15 ml/min collision gas flow rate. The collision gas used high-purity argon, and the rest of the gas paths used high-purity nitrogen. The data were analyzed by MassLynx software (Waters Co., Ltd., USA).

Statistical analysis. Data were analyzed with IBM® SPSS® 19.0 (IBM, USA) expressing as mean ± standard deviation. A one-way ANOVA was used to compare means. Statistical significance was determined by a *p*-value of less than 0.05.

Table II
Specific gene primers used for qRT-PCR.

Gene	Primers	Length (bp)
IL-6	F: CCCCAATTTCCAATGCTCTCC R: CGCACTAGGTTTGCCGAGTA	141
IL-10	F: TTAAAGGGTTACTTGGGTTGCC R: AATGCTCCTTGATTTCTGGGC	106
IL-1β	F: TCAAATCTCGCAGCAGCACATC R: CGTCACACACCAGCAGGTTATC	206
GAPDH	F: CCTCGTCCCGTAGACAAAATG R: TGAGGTCAATGAAGGGGTCGT	133

Results

Isolation and identification of isolates from human feces. Twenty-three isolates were obtained from human feces samples and confirmed by 16S rDNA sequencing. The sequences of isolates were uploaded to the NCBI database, and the accession number of all isolates are listed in Table III. The similarity of each isolate is over 99%. Eighteen strains were *L. fermentum*, and five strains were *L. plantarum*.

Inhibitory activities to α -amylase and α -glucosidase. **The inhibitory activities to α -amylase and α -glucosidase of the 23 isolates were determined** (Table IV). The α -amylase inhibitory activities of *L. plantarum* ranged from $17.33 \pm 5.03\%$ to $62.29 \pm 0.44\%$; the highest value was observed for *L. plantarum* 22. The α -amylase inhibitory activities of *L. fermentum* ranged from $16.84 \pm 8.34\%$ to $58.40 \pm 1.68\%$; the highest value was recorded for *L. fermentum* 11, followed by *L. fermentum* 305 ($57.48 \pm 5.04\%$). The α -glucosidase inhibitory activities of *L. plantarum* ranged from $11.45 \pm 4.18\%$ to $15.32 \pm 0.89\%$; the highest was for *L. plantarum* 25, followed by *L. plantarum* 22 ($14.89 \pm 0.38\%$). The α -glucosidase inhibitory activities of *L. fermentum* ranged

from $16.84 \pm 8.34\%$ to $58.40 \pm 1.68\%$, with the highest value for *L. fermentum* 11, followed by *L. fermentum* 305 ($57.48 \pm 5.04\%$). Based on the results of the inhibitory activities of the isolates to α -glucosidase and α -amylase, four potential anti-diabetic strains (*L. plantarum* 22, *L. plantarum* 25, *L. fermentum* 11, and *L. fermentum* 305) were selected due to high inhibitory activities to α -amylase and α -glucosidase. The viable counts of the two isolates of *L. plantarum* and the two isolates of *L. fermentum* were $1.22 \pm 0.47 \times 10^9$ CFU/ml, $1.84 \pm 0.70 \times 10^9$ CFU/ml, $2.55 \pm 0.30 \times 10^9$ CFU/ml, and $2.04 \pm 0.51 \times 10^9$ CFU/ml, respectively.

Tolerance to simulated digestion test and adhesion to Caco-2 cell. The survival rate of the four strains is shown in Table V. The survival rate of the strains after the simulated gastrointestinal digestion test were $12.42 \pm 2.84\%$, $9.10 \pm 1.12\%$, $5.86 \pm 0.52\%$, and $8.82 \pm 2.50\%$, respectively. The adhesion rates of *L. plantarum* 22 and *L. plantarum* 25 were $6.94 \pm 0.27\%$ and $6.91 \pm 0.11\%$, respectively, while the adhesion rates of *L. fermentum* 11 and *L. fermentum* 305 were $6.09 \pm 0.39\%$ and $6.37 \pm 0.28\%$, respectively.

Inhibitory activities to α -glucosidase and α -amylase of *Lactobacillaceae* powders. Table VI shows that the

Table III
Identification of *Lactobacillaceae* isolated based on 16S rDNA sequences
and gene accession number assigned by NCBI.

Isolate number	Species	GenBank accession number	Similarity (%)
4	<i>Limosilactobacillus fermentum</i>	MT482589	99.73
5	<i>Limosilactobacillus fermentum</i>	MT482590	99.93
6	<i>Limosilactobacillus fermentum</i>	MT482591	99.93
8	<i>Lactiplantibacillus plantarum</i>	MT482586	99.93
10	<i>Lactiplantibacillus plantarum</i>	MT482587	99.80
11	<i>Limosilactobacillus fermentum</i>	MT463724	99.93
12	<i>Limosilactobacillus fermentum</i>	MT482592	99.93
13	<i>Limosilactobacillus fermentum</i>	MT482593	99.93
14	<i>Limosilactobacillus fermentum</i>	MT482594	99.93
16	<i>Limosilactobacillus fermentum</i>	MT482595	99.93
18	<i>Limosilactobacillus fermentum</i>	MT482596	99.93
20	<i>Lactiplantibacillus plantarum</i>	MT482588	99.93
22	<i>Lactiplantibacillus plantarum</i>	MT463726	99.86
25	<i>Lactiplantibacillus plantarum</i>	MT463727	99.86
26	<i>Limosilactobacillus fermentum</i>	MT482597	99.93
27	<i>Limosilactobacillus fermentum</i>	MT482598	99.93
28	<i>Limosilactobacillus fermentum</i>	MT482599	99.93
29	<i>Limosilactobacillus fermentum</i>	MT482600	99.93
30	<i>Limosilactobacillus fermentum</i>	MT482601	99.93
33	<i>Limosilactobacillus fermentum</i>	MT482602	99.93
38	<i>Limosilactobacillus fermentum</i>	MT482603	99.79
39	<i>Limosilactobacillus fermentum</i>	MT482604	99.86
305	<i>Limosilactobacillus fermentum</i>	MT463725	99.30

Table IV
The inhibitory activities to α-amylase and α-glucosidase, and the viable count of the isolates.

	Isolate	Viable bacteria count (10 ⁹ CFU/ml)	α-amylase inhibitory activities (%)	α-glucosidase inhibitory activities (%)
<i>Lactiplantibacillus plantarum</i>	8	1.71 ± 0.47 ^{ab}	49.93 ± 2.06 ^c	12.39 ± 3.05 ^a
	10	2.61 ± 0.38 ^a	56.72 ± 8.78 ^{ab}	12.60 ± 3.22 ^a
	20	2.29 ± 0.73 ^{ab}	17.33 ± 5.03 ^d	11.45 ± 4.18 ^a
	22	1.22 ± 0.47 ^b	62.29 ± 0.44 ^a	14.89 ± 0.38 ^a
	25	1.84 ± 0.70 ^{ab}	51.81 ± 3.65 ^{bc}	15.32 ± 0.89 ^a
<i>Limosilactobacillus fermentum</i>	4	2.11 ± 0.64 ^b	20.96 ± 4.95 ^f	28.51 ± 5.73 ^b
	5	2.15 ± 0.25 ^b	16.84 ± 8.34 ^f	15.91 ± 0.91 ^d
	6	2.93 ± 0.28 ^{ab}	44.94 ± 3.72 ^c	19.33 ± 1.02 ^{cd}
	11	2.55 ± 0.30 ^b	58.40 ± 1.68 ^a	52.63 ± 3.07 ^a
	12	0.93 ± 0.66 ^c	44.05 ± 4.51 ^c	17.98 ± 9.09 ^{cd}
	13	2.85 ± 0.69 ^{ab}	51.86 ± 4.20 ^b	17.60 ± 1.84 ^{cd}
	14	2.96 ± 0.52 ^{ab}	37.19 ± 7.29 ^d	4.93 ± 0.94 ^e
	16	3.92 ± 0.38 ^a	32.29 ± 9.59 ^{de}	15.35 ± 8.28 ^d
	18	2.16 ± 0.80 ^b	27.35 ± 7.43 ^e	15.94 ± 1.38 ^d
	26	2.53 ± 0.98 ^b	49.95 ± 6.54 ^{bc}	15.60 ± 2.62 ^d
	27	2.49 ± 0.92 ^b	41.39 ± 2.52 ^{cd}	20.83 ± 4.58 ^c
	28	3.59 ± 0.50 ^{ab}	47.92 ± 3.62 ^{bc}	20.53 ± 0.17 ^c
	29	2.23 ± 0.67 ^b	46.74 ± 3.31 ^{bc}	4.99 ± 0.56 ^e
	30	2.41 ± 1.43 ^b	55.38 ± 0.48 ^{ab}	15.69 ± 1.93 ^d
	33	1.70 ± 0.90 ^{bc}	47.49 ± 2.27 ^{bc}	14.19 ± 2.13 ^d
	38	3.16 ± 0.77 ^{ab}	50.59 ± 3.12 ^{bc}	8.10 ± 4.18 ^e
	39	1.89 ± 0.59 ^{bc}	37.77 ± 8.18 ^d	5.24 ± 0.89 ^e
	305	2.04 ± 0.51 ^{bc}	57.48 ± 5.04 ^{ab}	51.79 ± 1.13 ^e

^{a-f} – means with different superscripts within the same row differ (*p* < 0.05)

Table V
Simulated digestion test and adhesion rates.

Isolate	Survival rate (%)	Adhesion rate (%)
<i>Lactiplantibacillus plantarum</i> 22	12.42 ± 2.84	6.94 ± 0.27
<i>Lactiplantibacillus plantarum</i> 25	9.10 ± 1.12	6.91 ± 0.11
<i>Limosilactobacillus fermentum</i> 11	5.86 ± 0.52	6.09 ± 0.39
<i>Limosilactobacillus fermentum</i> 305	8.82 ± 2.50	6.37 ± 0.28

optimal condition of α-amylase inhibitory activities was A₁B₁C₁D₁ (the optimal concentration of each strain in *Lactobacillaceae* powders was 10¹⁰ CFU/ml). The maximal inhibitory activity to α-amylase was 93.18 ± 1.19%. The effect of the factors in descending order was A > D > B > C. A, B, and D were significant factors, while C was a non-significant factor. *L. fermentum* strains 11 and 305 and *L. plantarum* 25 were significant factors, while *L. plantarum* 22 was a non-significant factor.

As was shown in Table VII, the inhibitory activities to α-glucosidase were most affected by *L. fermentum* 305, followed by *L. fermentum* 11, *L. plantarum* 22, and *L. plantarum* 25. The optimal conditions of α-glucosidase inhibitory activities were A₁B₁C₁D₁, and

the maximal inhibitory activities to α-glucosidase was 75.33 ± 2.89%. The effect of the factors in descending order was B > A > C > D. A, B, C, and D were significant factors. The optimal concentration of each strain in *Lactobacillaceae* powders was 10¹⁰ CFU/ml, and each strain was a significant factor.

Effects of the *Lactobacillaceae* mixture on body weight and fasting blood glucose in mice. The BW of the mice was significantly increased in the M and ML group compared with the N group in week 12, respectively (Fig. 1). Compared to the M group, the BW of the mice decreased slightly in the ML group in week 12. FBG was significantly increased in the M group compared with the N group in week 12. As compared to the

Table VI
Orthogonal test of the inhibitory activities to α -amylase of *Lactobacillaceae* powders.

Trial	A	B	C	D	Inhibitory activity (%)
1	1	1	3	2	70.00
2	1	2	1	1	82.50
3	1	3	2	3	38.00
4	2	1	2	1	71.50
5	2	2	3	3	7.50
6	2	3	1	2	31.00
7	3	1	1	3	43.00
8	3	2	2	2	30.00
9	3	3	3	1	25.50
K ₁	63.50	61.50	52.17	59.83	
K ₂	36.67	40.00	46.33	43.67	
K ₃	32.83	31.50	34.33	29.50	
R	30.67	30.00	17.84	30.33	
Optimum technologies	A ₁ B ₁ C ₁ D ₁				93.18 ± 1.19
SS	0.334	0.294	0.096	0.279	
df	2	2	2	2	
F	10.969	9.637	3.156	9.143	
Significance	*	*		*	

R-value is positively correlated with inhibitory activity.
* – means significant factor

Table VII
Orthogonal test of *Lactobacillaceae* powders' inhibitory activities to α -glucosidase.

Trial	A	B	C	D	Inhibitory activity (%)
1	1	1	3	2	73.57
2	1	2	1	1	63.28
3	1	3	2	3	54.32
4	2	1	2	1	71.93
5	2	2	3	3	35.05
6	2	3	1	2	62.74
7	3	1	1	3	64.79
8	3	2	2	2	24.18
9	3	3	3	1	54.39
K ₁	63.72	70.10	63.60	63.20	
K ₂	56.57	40.84	53.50	53.50	
K ₃	47.79	57.15	51.39	51.39	
R	15.94	29.26	12.22	11.81	
Optimum technologies	A ₁ B ₁ C ₁ D ₁				75.33 ± 2.89
SS	0.076	0.258	0.057	0.048	
df	2	2	2	2	
F	74.785	252.387	55.709		
Significance	*	*	*	*	

R-value is positively correlated with inhibitory activity.
* – means significant factor

M group, the FBG of the mice significantly decreased in the ML group in week 12. During the first four weeks of the treatment, the FBG of the mice in the M group increased and then remained at a higher level. Interestingly, the *Lactobacillaceae* mixture-treated db/db mice showed ameliorated FBG from weeks 6 to 12 ($p < 0.05$). In week 12, the ML group displayed an FBG of 22.4% lower than the M group.

Effects of the *Lactobacillaceae* mixture on the liver, kidney, pancreas, and colon in mice. In the N group,

the liver, kidney, pancreas, and colon structures were well-organized and showed no pathological signs. Compared to the N group, in the M group, the following was observed: 1) many vacuoles, irregularly arranged and disordered cells in the liver (Fig. 2a); 2) degeneration of glomerular and tubular structures and fat accumulation in the kidney (Fig. 2b); 3) islet atrophy and focal necrosis in the pancreas (Fig. 2c); and 4) a thinner intestinal wall, degeneration of structures and decrease of cells (Fig. 2d). However, the *Lactobacillaceae* culture

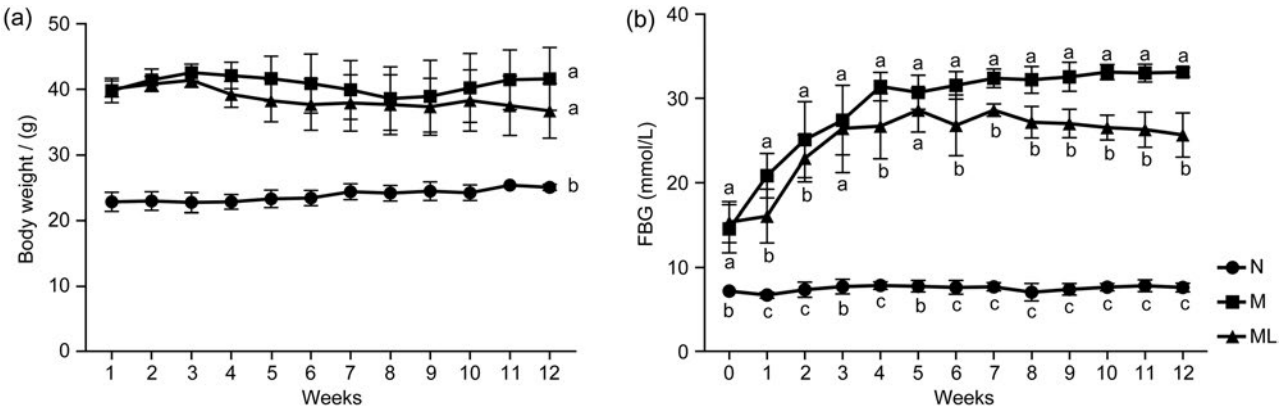


Fig 1. Changes of body weight (a) and fasting blood glucose (FBG) levels (b) in mice. Significant differences exist between values with different letters ($p < 0.05$).

N – Control group, wt/wt mice; M – model group, db/db mice;
ML – *Lactobacillaceae*-treated group, db/db mice with the *Lactobacillaceae* mixture intervention

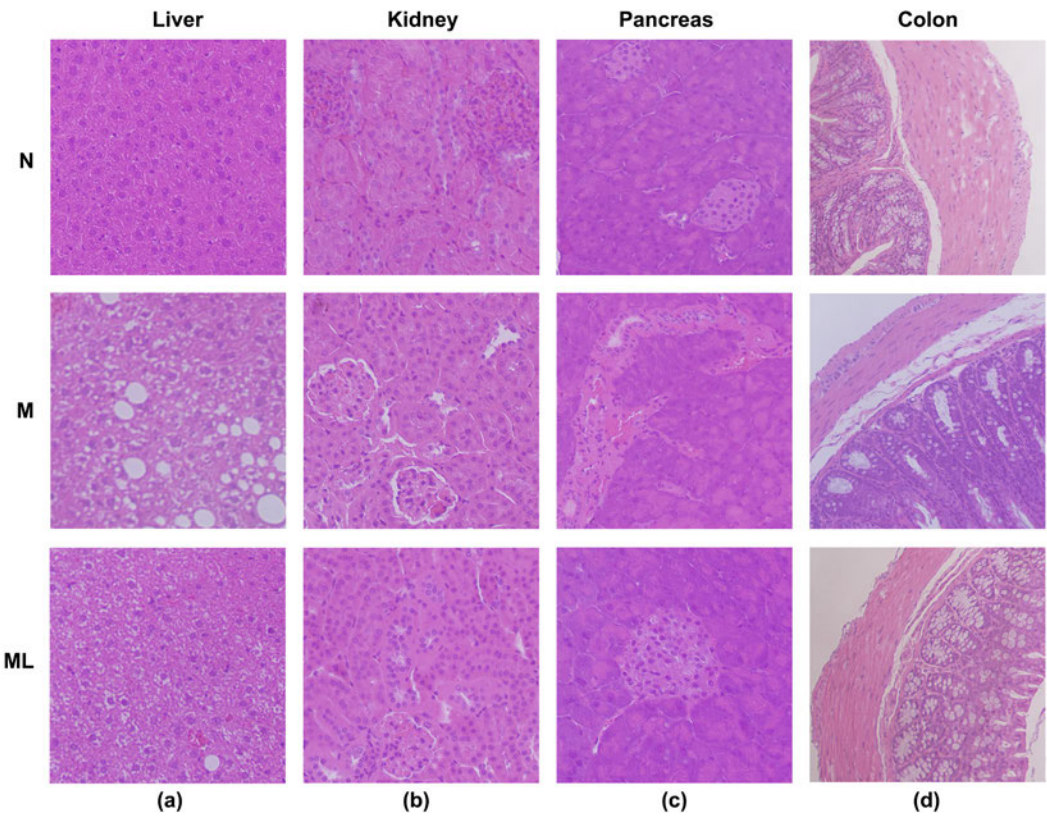


Fig 2. Morphological alterations in the liver (a), kidney (b), pancreas (c), and colon (d).

N – Control group, wt/wt mice; M – model group, db/db mice; ML – *Lactobacillaceae*-treated group, db/db mice with the *Lactobacillaceae* mixture intervention

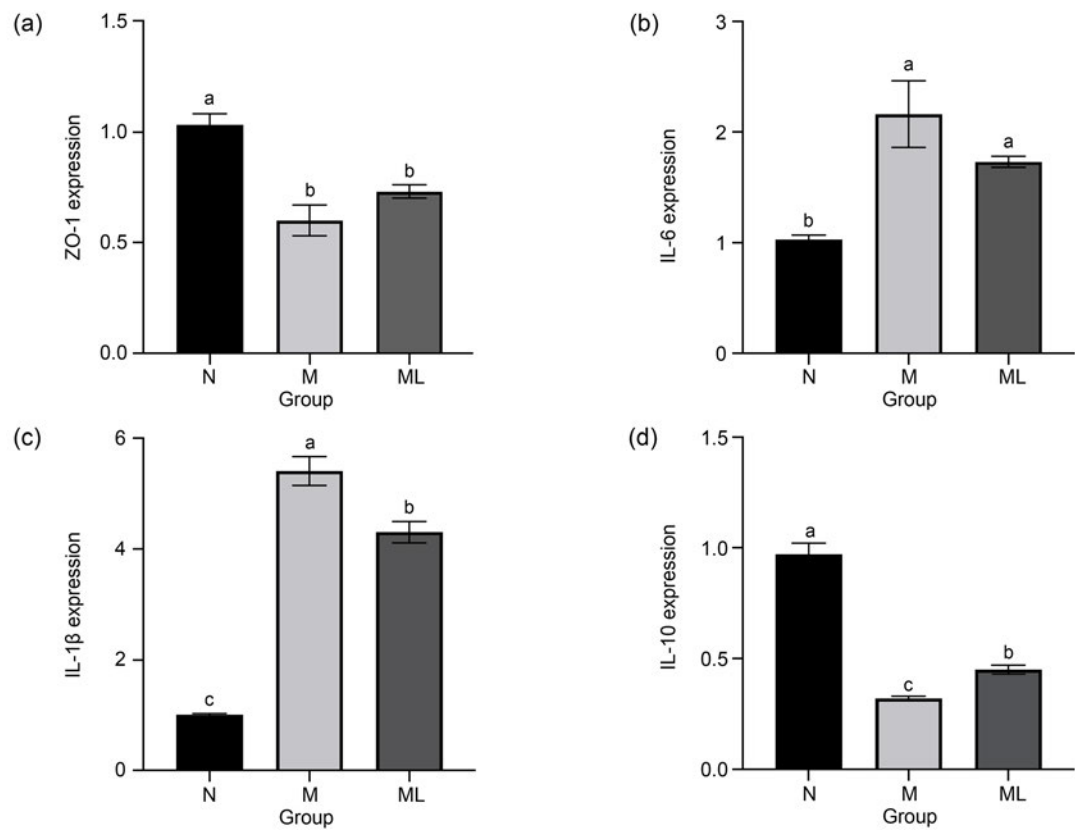


Fig 3. Colon inflammatory factor mRNA expression. Significant differences exist between values with different letters ($p < 0.05$).
N – Control group, wt/wt mice; M – model group, db/db mice; ML – *Lactobacillaceae*-treated group, db/db mice with the *Lactobacillaceae* mixture intervention

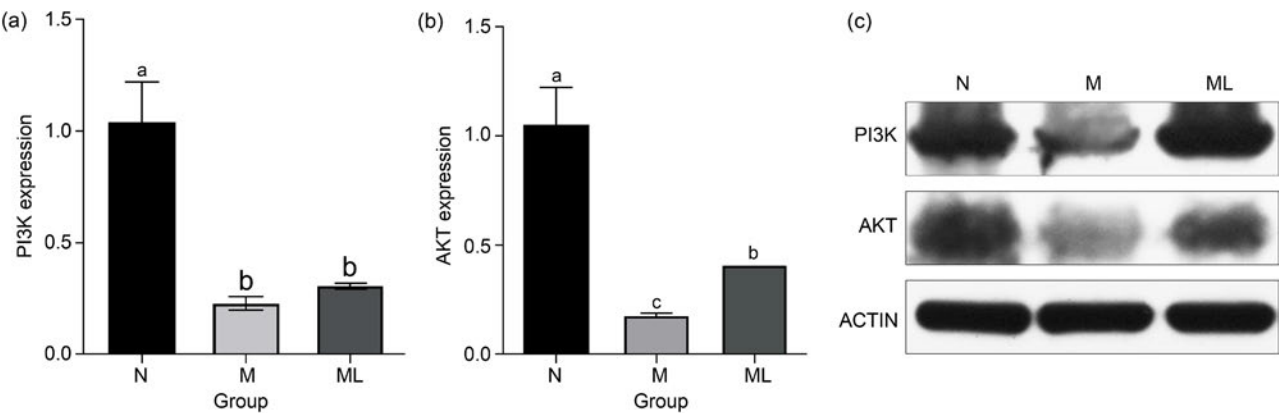


Fig 4. PI3K, AKT protein expression electropherogram, and protein relative expression. Significant differences exist between values with different letters ($p < 0.05$).
N – Control group, wt/wt mice; M – model group, db/db mice; ML – *Lactobacillaceae*-treated group, db/db mice with the *Lactobacillaceae* mixture intervention

revealed favorable influences on the injuries of liver, kidney, pancreas, and colon respectively.

Effects of the *Lactobacillaceae* mixture on the expression of inflammatory factors and claudin mRNA in the colon of mice. As shown in Fig. 3, the mRNA expression levels of IL-6 and IL-1 β were significantly increased. In contrast, the mRNA expression levels of IL-10 and ZO-1 were significantly decreased in the M group compared with the N group. There was a sig-

nificant increase in the mRNA expression levels of IL-10 in the ML group compared to the M group, while the mRNA expression levels of IL-1 β significantly decreased. *Lactobacillaceae* isolates slightly increased the mRNA expression levels of ZO-1 but decreased that of IL-6.

Effects of the *Lactobacillaceae* mixture on the expression of PI3K and AKT in mice. The protein expression levels of PI3K and AKT in the liver are shown in Fig. 4. Compared with the N group, the protein

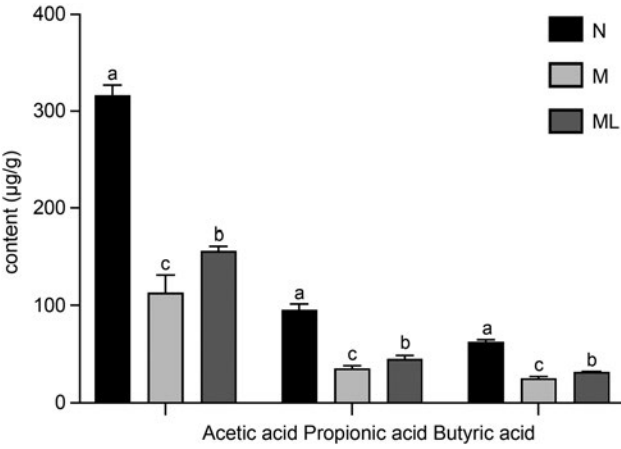


Fig 5. SCFAs content of mice in each group. Significant differences exist between values with different letters ($p < 0.05$).
N – Control group, wt/wt mice; M – model group, db/db mice;
ML – *Lactobacillaceae*-treated group, db/db mice with the *Lactobacillaceae* mixture intervention

expression levels of PI3K and AKT in the M group were significantly decreased. There was a significant increase in the protein expression levels of AKT in the ML group compared with the M group. In contrast, the protein expression levels of PI3K increased, but not significantly.

Effects of the *Lactobacillaceae* mixture on the level of SCFAs in mice. The contents of SCFAs in each group are shown in Fig. 5. Acetic acid, propionic acid, and butyric acid contents in the M group were significantly lower than in the N group. There was a significant increase in acetic acid and butyric acid contents in the ML group compared to the M group. however, no significant difference was detected in the contents of propionic acid between the M and ML group.

Discussion

Twenty-three isolates were obtained from the adult feces and identified as *L. fermentum* or *L. plantarum* by 16S rDNA sequencing. Similarly, *L. plantarum* and *L. fermentum* were also isolated from infant feces (Jomehzadeh et al. 2020), similar to our results. Additionally, there are different strains isolated from human feces in other reports. *Lactocaseibacillus rhamnosus* 4B15 and *Lactobacillus gasseri* 4M13 were isolated from infant feces; *Levilactobacillus brevis* 205, *Lactobacillus mocusae* 226, and *Lactocaseibacillus casei* 375 were isolated from human feces (Oh et al. 2018; Beglari et al. 2022).

It has been shown that the inhibition of α -amylase can reduce postprandial glucose levels (Saleem et al. 2018). In this study, a similar theory was applied to *in vitro* antidiabetic. α -Glucosidase is a critical enzyme responsible for converting oligosaccharides and disaccharides into monosaccharides in the gut (Schloesser

et al. 2017). *L. plantarum* isolates 22 and 25, and *L. fermentum* isolates 11 and 305 were selected from 23 strains of *Lactobacillaceae* identified by 16S rDNA sequencing in human feces. They were chosen due to their higher *in vitro* antidiabetic activity. Their inhibitory activities to α -amylase were $62.29 \pm 0.44\%$, $51.81 \pm 3.65\%$ and $58.40 \pm 1.68\%$, $57.48 \pm 5.04\%$, respectively. Their inhibitory activities to α -glucosidase were $14.89 \pm 0.38\%$, $15.32 \pm 0.89\%$ and $52.63 \pm 3.07\%$, $51.79 \pm 1.13\%$, respectively. Chen et al. (2014) found that inhibitory activity to α -glucosidase of *L. rhamnosus* Z7 isolated from human feces was about 29.21%. Panwar et al. (2014) reviewed that the amylase and maltase inhibitory rates of *Lactobacillaceae* strains isolated from infant feces were 19.4–89.4% and 15–76%, respectively. By comparison, the four strains of *Lactobacillaceae* in our study had excellent inhibitory activity to α -amylase and α -glucosidase. Therefore, these *Lactobacillaceae* strains were used as the potential strains against diabetes in this study.

Tolerance in the gastrointestinal tract is one of the probiotics’ most important features. The potential probiotics can tolerate the digestive tract environment to improve the physical condition of hosts (Salehizadeh et al. 2020). The survival rate of four of *Lactobacillaceae* strains after the simulated gastric digestion test ranged from 5.86% to 12.42% in this research. Zhu et al. (2015) indicated that the survival rate of some strains, including *Bifidobacteria* and *Lactobacillaceae*, was less than 10% after a sequential simulated gastric digestion test. Belguesmia et al. (2016) showed that the survival rate of some *Lactobacillaceae* was about 10%. These results were similar to ours. Probiotic strains need to adhere to intestinal epithelium to exert their beneficial effects. It is one of the most important criteria when selecting probiotic strains (Kim et al. 2018). Many scholars have investigated the adhesion of probiotics to Caco-2 cells. Huang et al. (2013) showed that the adhesion rate to Caco-2 cell of some *Lactobacillaceae* (*Ligilactobacillus animalis*, *L. reuteri*, *Lactobacillus johnsonii*, *L. plantarum*, *Lactobacillus amylovorus*, *L. acidophilus*, *L. gasseri*, *L. casei*, *L. salivarius*, and *Latilactobacillus sakei*) isolated from pig feces ranged from 2.25% to 20.16%. Ramos et al. (2013) reported that the adhesion rate to Caco-2 cells of some *Lactobacillaceae* strains were less than 1.8%. In this study, the strains showed a higher adhesion rate to Caco-2 cells ranging from 6.09% to 6.94%. From the results, we conclude that the four *Lactobacillaceae* strains have the potential to survive in the gastrointestinal environment and multiply in the intestinal tract.

Probiotics in the intestines have synergistic and complementary effects, so the benefits of mixed probiotic strains for humans are believed to be better than single strains (Al-Muzafar and Amin 2017). In this study, the orthogonal tests of the *Lactobacillaceae* mixture showed that the maximum inhibitory activities to

α -amylase and α -glucosidase were $93.18 \pm 1.19\%$ and $75.33 \pm 2.89\%$, respectively. Our results showed that the anti-diabetic effect *in vitro* was significantly improved after the combination of these four strains. It provided a good start for our further experiments.

The presence of hyperglycemia characterizes T2DM. It is crucial to control FBG levels in treating and preventing T2DM. The FBG level was significantly higher in the M group than in the N group in this research. The maximum FBG level in the M group was 33.11 ± 0.89 mmol/L. After 12 weeks of treatment, the FBG level was significantly reduced in the ML group compared with the M group. Similarly, Hsieh et al. (2020) reported that *L. salivarius* AP-32 and *L. reuteri* GL-104 improved T2DM by reducing FBG levels. Therefore, our *Lactobacillaceae* mixture may possess anti-hyperglycemic properties to alleviate the development of T2DM.

The liver is critical for glucose and lipid metabolism. Diabetes can cause liver damage and destroy liver function (Abo El-Nasr et al. 2020). Morphological alterations in the liver verified the severity of liver damage in mice with T2DM, and the *Lactobacillaceae* mixture significantly diminished liver damage. Hyperglycemia caused by disorders of glucose metabolism can damage the kidneys (Abo El-Nasr et al. 2020). According to the results of kidney morphological changes, it was found that the mice with T2DM had severely damaged kidney structures and had evidence of glomerular atrophies, and our *Lactobacillaceae* mixture was effective in inhibiting kidney damage. The pancreatic islets, the most important part of the pancreas, can release glucagon and insulin to balance blood glucose levels (Noguchi and Huising 2019). Mice with T2DM had severely damaged pancreatic tissue, and the number of normal islet cells was drastically reduced, adversely affecting glucose metabolism. Interestingly, the number of damaged islet cells in db/db mice treated with our *Lactobacillaceae* mixture was reduced, and the islet function improved. *Lactocaseibacillus paracasei* NL41 simultaneously alleviated liver, kidney, and pancreatic cell damage, similar to our results (Zeng et al. 2019).

Gut immunity and inflammation are crucial in metabolic diseases (Zhang et al. 2021). Vemuri et al. (2018) found that human-derived probiotics *L. acidophilus* DDS-1 and *B. animalis* ssp. *lactis* UABla-12 could induce human colon cancer cells to secrete IL-10. In this study, the reduction of the anti-inflammatory factor IL-10 and increase in colonic pro-inflammatory IL-1 β in the M group could be significantly attenuated by the intervention of the *Lactobacillaceae* mixture. It has been proposed that the *Lactobacillaceae* mixture improved intestinal health in mice with T2DM by alleviating inflammation in the gut.

The PI3K/AKT signaling pathway disorder is associated with high glucose concentrations, which is a criti-

cal cause of diabetes (Mohseni et al. 2021). *L. paracasei* TD062 could relieve glucose homeostasis by elevating AKT's expression to prevent the development of T2DM (Dang et al. 2018). The level of AKT significantly increased in db/db mice after being treated with the *Lactobacillaceae* mixture, while the level of PI3K increased with no significant difference. Therefore, the *Lactobacillaceae* mixture could alleviate T2DM by improving the PI3K/AKT signaling pathway.

SCFAs are essential for supporting the structure and performance of epithelial cells (Liu et al. 2018). Meanwhile, SCFAs are beneficial for regulating glucose homeostasis and insulin secretion (Portincasa et al. 2022). Acetic acid can stimulate islet B cells, promote insulin secretion, adjust blood glucose and lipids, and maintain insulin sensitivity (Li et al. 2015). Butyric acid is metabolized by hindgut cells as a significant energy source and is transported to the liver for use in different biosynthetic pathways (Liu et al. 2018). Moreover, SCFAs can regulate the pH of the intestine to prevent harmful bacteria growth (Liu et al. 2018). Li et al. (2016) informed that *L. plantarum* X1 had the hypoglycemia ability associated with increasing acetic acid and butyric acid content in the feces of diabetic mice. The reduction of acetic acid and butyric acid in the M group could be significantly attenuated by the intervention of the *Lactobacillaceae* mixture in this work. The *Lactobacillaceae* mixture may improve the hyperglycemia symptoms of mice with T2DM by increasing the level of SCFAs in the gut.

Conclusions

In conclusion, the *Lactobacillaceae* mixture including *L. plantarum* 22, *L. plantarum* 25, *L. fermentum* 11, and *L. fermentum* 305 was isolated from human feces and identified as potential probiotics to improve T2DM. The *Lactobacillaceae* mixture relieved T2DM by lowering blood glucose, increasing the level of SCFAs, improving the PI3K/AKT signaling pathway, ameliorating secretion of inflammatory factors, and relieving the liver, kidney, pancreas, and colon damage in db/db mice. The *Lactobacillaceae* mixture could be a reference for developing non-toxic treatment options with hypoglycemic effects without side effects. Further safety assessments are warranted, and clinical trials should be performed in appropriate diabetic patients.

Ethical statement

Animal experiment followed the recommendations of the Animal Welfare Committee of Beijing Union University (Beijing, China). The protocol was approved by the Animal Welfare Committee of Beijing Union University (protocol code: 20200802).

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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