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Study on the effect of licochalcone A on intestinal flora in type 2 diabetes mellitus mice based on 16S rRNA technology†

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Licorice, has a long history in China where it has various uses, including as a medicine, and is often widely consumed as a food ingredient. Licorice is rich in various active components, including polysaccharides, triterpenoids, alkaloids, and nucleosides, among which licochalcone A (LicA) is an active component with multiple physiological effects. Previous studies from our research group have shown that LicA can significantly improve glucose and lipid metabolism and related complications in Type 2 diabetes mellitus (T2DM) mice. However, research on the mechanism of LicA in T2DM mice based on intestinal flora has not been carried out in depth. Therefore, in this study, LicA was taken as the research object and the effects of LicA on glucose and lipid metabolism and intestinal flora in T2DM mice induced by streptozotocin (STZ)/high-fat feed (HFD) were explored. The results indicated that LicA could reduce serum TC, TG, and LDL-C levels, increase HDL-C levels, reduce blood glucose, and improve insulin resistance and glucose tolerance. LicA also alleviated pathological damage to the liver. The results also showed that LicA significantly affected the intestinal microbiota composition and increased the α diversity index. β Diversity analysis showed that after the intervention of LicA, the composition of intestinal flora was significantly different from that in the T2DM model group. Correlation analysis showed that the changes in glucose and lipid metabolism parameters in mice were significantly correlated with the relative abundance of *Firmicutes*, *Bacteroidetes*, *Helicobacter*, and *Lachnospiraceae* ($p < 0.01$). Analysis of key bacteria showed that LicA could significantly promote the growth of beneficial bacteria, such as Bifidobacterium, Turicibacter, Blautia, and Faecococcus, and inhibit the growth of harmful bacteria, such as Enterococcus, Dorea, and Arachnococcus. In conclusion, it was confirmed that LicA reversed the imbalanced intestinal flora, and increased the richness and diversity of the species in T2DM mice.

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Introduction

Diabetes mellitus is a disease caused by the dysfunction of pancreatic β-cell secretion, resulting in metabolic disorders, such as carbohydrates and fat.[1,2] Insulin resistance (IR) and elevated blood sugar levels are the main clinical manifestations of diabetes. Diabetes patients present various diabetes symptoms, so diabetes is given different names.[3,4] Type 1 diabetes mellitus, gestational diabetes mellitus, and type 2 diabetes mellitus (T2DM) are the main types of diabetes mellitus.[5,6] According to the International Diabetes Federation (IDF), the number of people with diabetes is expected to reach 700.2 million by 2045. China is a disaster area of diabetes, and the number of diabetes patients in China has ranked first in the world.[7] The incidence rate of diabetes in China is about 10%, and the number of diabetes patients has reached 114 million, accounting for one-third of the total number of diabetes patients in the world.[7] In addition, the vast majority of patients with diabetes (>90%) belong to T2DM.[5,8] At present, Western drugs are still the main means of clinical intervention in T2DM, such as metformin and subcutaneous injection of insulin, but there are some problems, such as strict control of blood sugar, greater fluctuation of blood sugar, and gastrointestinal discomfort.[9–12] Among many related factors, the imbalance of intestinal flora and the occurrence and development of T2DM have attracted more and more attention from the academic community.

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Studies have shown that there is a certain relationship between intestinal flora and T2DM, and the imbalance of intestinal flora may be involved in the pathogenesis of T2DM.[13] The study found that the intestinal flora, as a new target for the treatment of diabetes, has a good role in treating and alleviating hyperglycemia symptoms.[14,15] The types and abundance of intestinal microbiota in patients with T2DM are significantly different from those in normal controls.[16–19] The intestinal bacteria in the healthy human gut, weighing up to 2 kg, can affect host metabolism, inhibit the invasion of pathogenic bacteria, and the development of immune diseases, which is called the "second genome".[20] The intestinal bacteria can affect blood glucose homeostasis through multiple metabolic pathways, and induce diabetes and complications. It is generally believed that the occurrence of T2DM is one of the results of intestinal microbial disorder caused by an over-nutrition diet. The excessive intake of salt, sugar, fat, and other nutritious foods has a destructive impact on the diversity and stability of the flora, which is characterized by the reduction of beneficial flora and the increase of conditionally pathogenic flora, leading to the occurrence of IR and T2DM.

How to treat T2DM and daily health care, especially to find and develop effective, safe, and convenient hypoglycemic substances from natural plants, has become an important topic for medical workers at home and abroad. As an excellent natural plant resource, licorice has been widely used in the field of medicine. Licorice has been used by human beings for nearly 4000 years. In the Compendium of Materia Medica of Shizhen Li in the Ming Dynasty, licorice was listed as the first taste of more than 1000 kinds of traditional Chinese medicines. Since ancient times, licorice has been widely used for medicine, and has the honor of "national elder".[21,22] Furthermore, licorice is also a kind of medicine and food homologous plant.[23] Licorice is used in a variety of common cooking, such as braising, stewing, boiling, and steaming, as a condiment to add fragrance and remove fishiness.[23] Licorice is also recognized as a food additive in the EU, the United States, China, and other countries. It mainly contains glycyrrhizic acid, glycyrrhetinic acid, flavonoids, saponins, and other components. LicA, as a flavonoid in licorice, has an obvious antibacterial, antioxidant, anti-inflammatory, anti-tumor, immune promotion, and other activities, with high potential economic value.[24–27]

Meanwhile, our previous study showed that LicA could significantly improve glucose and lipid metabolism and related complications in T2DM mice.[28,29] However, there are few reports on the effect of LicA on the intestinal flora of patients with T2DM. Based on previous studies,[28,29] it is still necessary to further study whether LicA can regulate intestinal flora structure and its association with anti-T2DM. Therefore, the mice model of T2DM was established in this experiment to evaluate the effect of LicA on glucose and lipid metabolism in T2DM mice. High-throughput sequencing technology was introduced to explore the relationship between the pathogenesis of T2DM and intestinal microorganisms, and the regulatory effect of LicA on the intestinal flora of the host, evaluate its impact on the composition, and structure of intestinal flora, and preliminarily explore the possible mechanism of the anti-T2DM effect of LicA.

Materials and methods

Materials and reagents

LicA (purity > 95%) was obtained from licorice residues (as shown in ESI Fig. S1†). The chemical structure of the LicA is shown in Fig. S2.† Streptozotocin (STZ) was obtained from Sigma Corporation (USA). The Accu-Chek glucometer was obtained from Merck Sharp & Dohme Ltd, Hertfordshire, EN119BU, UK.

Animals

The male C57BL/6 mice with body weights of 20 ± 2 g were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University. The mice were raised in the clean-barrier Animal Laboratory of the experimental animal center of Shenyang Pharmaceutical University. The daily care and experimental conditions of animals shall refer to the environmental and facility standards for the experimental animals of the Ministry of the Health of the People's Republic of China. The animal experiments were approved by the animal ethics committee of Shenyang Pharmaceutical University, no. SYPU-IACUC-C2019-11-29-206.

T2DM induction

C57BL/6 mice of SPF grade were selected. After 1 week of adaptation to the basic diet, the mice were randomly divided into the control group and the high-fat feed (HFD) group. The control group was given the normal diet (n = 10, 4.7% fat, 57% carbohydrate, and 20% protein),[30] mice of the HFD group were given HFD (60% energy from fat, XTHF60, Xietong Pharmaceutical Bioengineering Co., Ltd, Jiangsu, China) every day for 6 weeks. After 6 weeks, the T2DM model was induced by intraperitoneal injection of streptozotocin (STZ) at 50 mg kg^{-1} for 3 consecutive days. After 72 h and 1 week, the fasting blood glucose (FBG) was measured. If the FBG of mice was greater than 11.1 mmol L^{-1}, the model was judged to be successful. Mice whose FBG levels were lower than 11.1 mmol L^{-1} were excluded. The successful mice were randomly divided into two groups: the model group (n = 10) and the LicA group (35 mg kg^{-1}, n = 10).

Experimental design

After the establishment of the T2DM model, the control group and model group were given normal saline by gavage. The LicA group was given 35 mg kg^{-1} intragastric administration of LicA, once a day. After 4 weeks of administration, the cecum contents of mice were collected aseptically. 6 replicates were taken from each group and placed into the sterilized centrifuge tube for bacterial flora analysis. The body weight and FBG of mice in each group were recorded weekly.

Oral glucose tolerance test (OGTT)

After the last drug administration, the mice were fasted for 12 h. The next day, the glucose solution was gavaged at 2.0 g kg^{-1}, and the blood was collected at 0, 30, 60, and 120 min to measure the blood glucose value and calculate the area under the curve (AUC).

Determination of metabolic parameters

The serum was separated, and triglyceride (TG), cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) contents in the serum were determined according to the steps of the kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Fasting serum insulin (FINS) was determined according to the instructions of the ELISA kit of Beijing Bioson Bioengineering Co., Ltd. The insulin sensitivity index Homeostatic index of insulin resistance (HOMA-IR) was calculated by the following method: HOMA-IR = FIN (mIU L^{-1}) × FBG (mmol L^{-1})/22.5.

Histopathological observation. The livers of each group were fixed in 10% formaldehyde solution, dehydrated, embedded in paraffin, sliced 3–5 μm thick, and stained with hematoxylin–eosin (HE). Pathological changes in the tissues were observed under a microscope.

Gut microbiota analysis. A commercial rapid DNA spin extraction kit (MP Biomedicals, Santa Ana, CA, USA) was used to extract the bacterial genomic DNA, and a NanoDrop spectrophotometer was used to quantify the extracted DNA according to its manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The 16S rDNA high-throughput sequencing was conducted on a Miseq platform (Illumina, San Diego, USA) at Personalbio Technology Co., Ltd in Shanghai, China. The library and sample were divided according to the index and barcode information of the original sequence of quality preliminary screening, and the barcode sequence was removed. Sequence denoising or OTU clustering is performed according to the QIIME2 dada2 analysis process. The specific composition of each sample (group) at the taxonomic levels of different species was displayed to understand the overall situation. According to the distribution of ASV/OTU in different samples, the Alpha diversity level of each sample was evaluated, and the sparse curve reflected whether the sequencing depth was appropriate. At the ASV/OTU level, the distance matrix of each sample was calculated, and the difference and significance of beta diversity between different groups were measured using a variety of unsupervised sequencing and clustering methods, combined with corresponding statistical testing methods. The correlation between intestinal flora and glycolipid metabolism parameters was analyzed using the Spearman coefficient. At the level of species taxonomic composition, a variety of unsupervised and supervised sequencing, clustering, and modeling methods, combined with corresponding statistical testing methods, were used to measure the differences in abundance composition of species among different groups and to identify marker species. Line discriminant analysis effect size (LEfSe) was used to analyze species with significant differences between groups.

Statistical analysis. GraphPad Prism 7.0 was employed to perform the analysis. For multiple group comparisons, a one-way analysis of variance and Tukey's multiple comparison tests were performed. The results are presented as mean ± standard deviation (SD). Differences between groups were represented by repeated one-way analysis of variance (ANOVA). $p < 0.05$ was statistically significant.

Results

Effects of LicA on FBG, body weight, and OGTT in the experimental mice

As shown in Fig. 1A, the FBG of mice in the T2DM model group was significantly increased compared with that in the control group ($p < 0.01$). At the end of week 4, the FBG in the LicA group was significantly lower compared with that in the model group ($p < 0.01$). As shown in Fig. 1B, compared to the control group, the weight of mice in the model group was significantly decreased ($p < 0.01$), and the intervention of LicA alleviated the phenomenon of weight loss in mice. As can be seen from Fig. 1C and D, the blood glucose value and AUC at each time point before and after glucose administration in the model group were significantly higher than those in the control group, with abnormal glucose tolerance. Compared with the model group, the blood glucose value, and AUC at each time point before and after glucose administration in the LicA group were significantly reduced ($p < 0.01$). The decrease of blood glucose in the LicA group at 2 h after the sugar load was statistically significant. The results showed that LicA could improve the impaired glucose tolerance of T2DM mice.

Effect of LicA on lipid metabolism in the experimental mice

It can be seen from Fig. 2 that compared with the control group, the contents of TC, TG, and LDL-C in the model group were significantly increased ($p < 0.01$), while the contents of HDL-C were significantly decreased ($p < 0.01$). Compared with the model group, the LicA group could significantly reduce the content of TC, TG, and LDL-C in the serum of mice ($p < 0.05$), and increase the content of HDL-C ($p < 0.05$). It was suggested that LicA could improve dyslipidemia in T2DM mice.

Effects of LicA on insulin and HOMA-IR in the experimental mice

As shown in Fig. 3, compared with the control group, the FINS in the model group was significantly decreased ($p < 0.01$), and the HOMA-IR was significantly increased ($p < 0.01$). Compared with the model group, the LicA group showed significantly increased FINS of the T2DM mice ($p < 0.01$) and decreased HOMA-IR.

Effect of LicA on liver histopathology in the experimental mice

As shown in Fig. 4, the results of HE staining in the liver show that the liver cells in the control group were uniform in size,

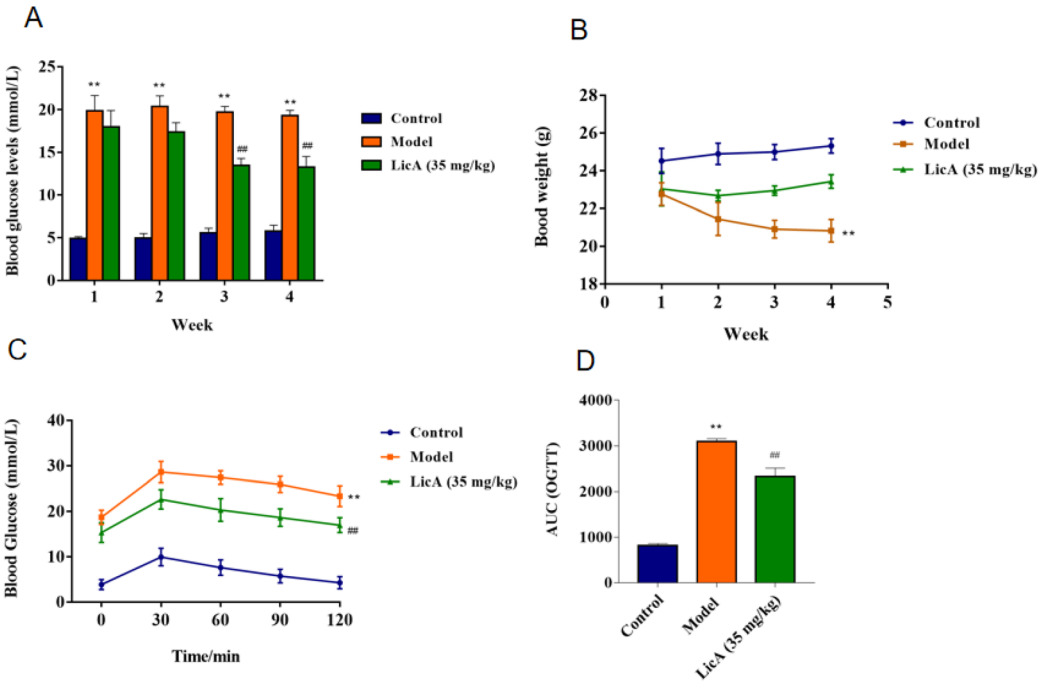

Fig. 1 Effects of LicA on FBG (A), body weight (B), and OGTT (C) of experimental mice. (D) Quantification of the AUC from the OGTT in C. Data are expressed as the mean ± standard deviation (SD) (n = 6). $^{**}p < 0.01$ compared with the control group. $^{\#\#}p < 0.01$ compared with the model group.

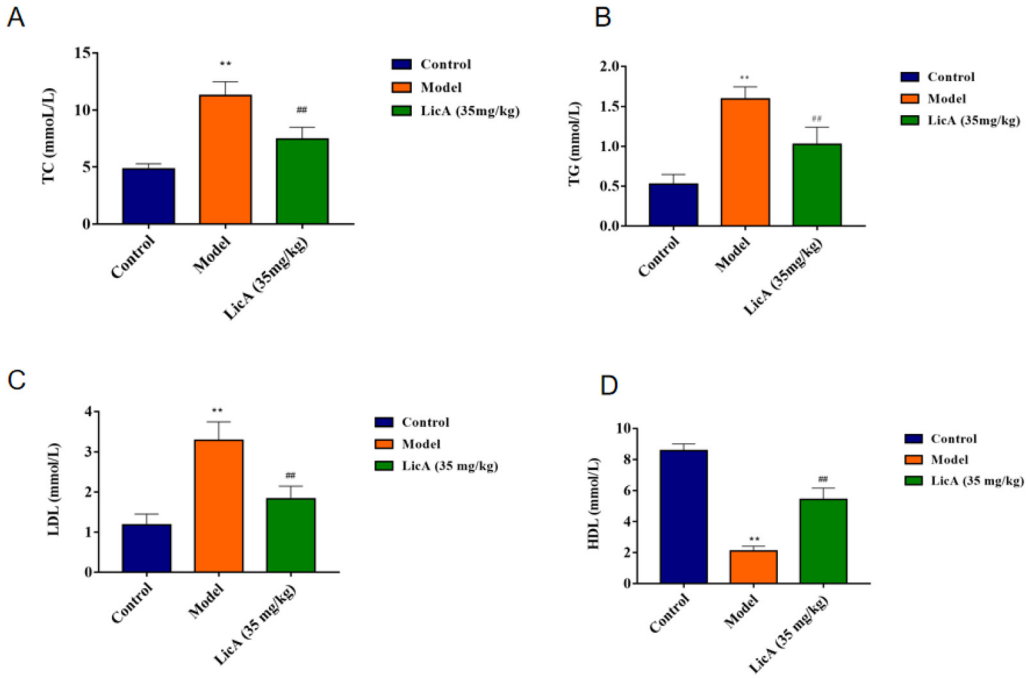

Fig. 2 Effect of LicA on blood lipid in T2DM mice. (A) Total cholesterol (TC; n = 6). (B) Triglyceride (TG; n = 6). (C) Low-density lipoprotein cholesterol (LDL-C; n = 6). (D) High-density lipoprotein cholesterol (HDL-C; n = 6). All date are expressed as mean ± S.D. $^{**}p < 0.01$ compared with the control group. $^{\#\#}p < 0.01$ compared with the model group.

the structure of hepatic lobules was clear, and there was no fibrous tissue proliferation. In the model group, the arrangement of hepatocytes was disordered, the outline of the cells was unclear, fat vacuoles, and droplets could be seen in the cytoplasm, and the degeneration of liver cells could be seen. Compared with the model group, after 4 weeks of LicA treat-

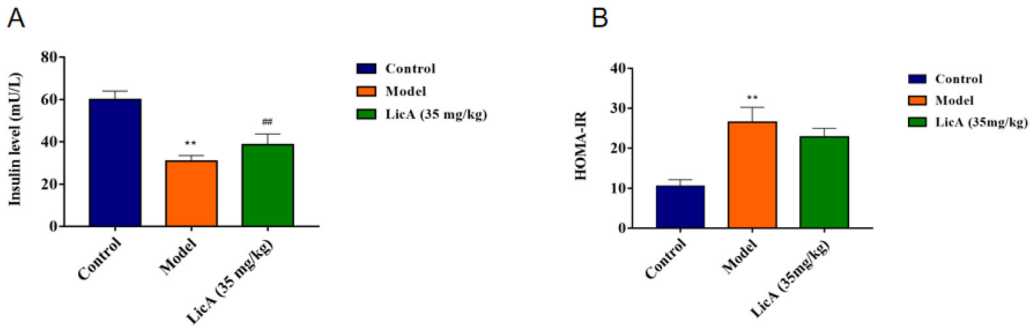

Fig. 3 Effects of LicA on insulin levels and HOMA-IR results. (A) Insulin levels of experimental mice. (B) HOMA-IR of experimental mice. Data are expressed as the mean ± standard deviation (SD) (n = 6). $^{**}p < 0.01$ compared with the control group. $^{\#\#}p < 0.01$ compared with the model group.

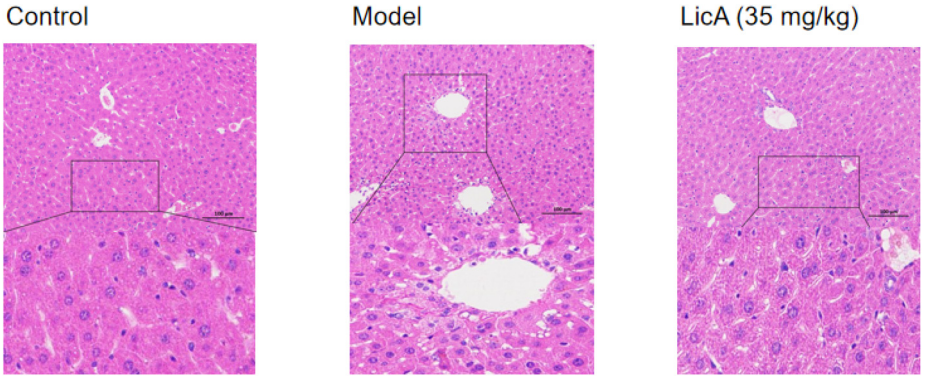

Fig. 4 Effects of LicA on histopathology in experimental mice. The liver tissues were stained with H&E and micrographs were taken at 200× magnification.

ment, the morphology and structure of hepatocytes were more complete, and vacuolar degeneration and lipid droplets were significantly reduced, which was improved.

Analysis of alpha diversity dilution curve

The dilution curve was used to randomly select a certain number of sequences from the sample and the Alpha diversity indexes corresponding to these sequences were calculated. The amount of extracted data was taken as the horizontal coordinate and the Alpha diversity index value as the vertical coordinate. The curve was drawn to determine whether the amount of sequencing data was sufficient according to whether the curve was gentle or not. It can be seen from Fig. 5 that the dilution curves of the three indexes tended to be flat, indicating that the number of sequencing data was large enough to reflect the vast majority of microbial diversity information in the sample.

Abundance distribution curve

Rank-abundance curves can explain species abundance and species evenness of samples.[31] In the horizontal direction, the width of the curve represents species abundance, the larger the range of the curve on the horizontal axis, the higher the abundance. The evenness of species is represented by the shape (smoothness) of the curve. The gentler the curve, the more uniform the distribution of the species. As shown in Fig. 6, in the horizontal direction, compared with the model group, the control group and LicA group have a larger span on the horizontal axis, indicating that their species richness is higher. In the vertical direction, the curves of the control group and LicA group were smoother than those of the model group, reflecting the more uniform distribution of species in the samples.

Effect of LicA on alpha diversity index of T2DM mice

In order to comprehensively assess the alpha diversity of microbial communities, the richness was represented by Chao[32] and Observed_species, and the diversity was represented by Shannon[33,34] and Simpson indices.[35] Then, the diversity based on evolution was characterized by the Faith_pd index,[36] the evenness was represented by the Pielou_e index,[37] and the coverage was represented by the Goods_coverage index.[38] As shown in Fig. 7, compared to the control group, the Observed_species index, Shannon index, chao1 index, Simpson index, and Faith_pd index of the T2DM mice were decreased, which was consistent with the existing research results.[39,40] This indicated that the intestinal flora of T2DM mice had low species richness and diversity, and the preventive administration of LicA could prevent this phenomenon.

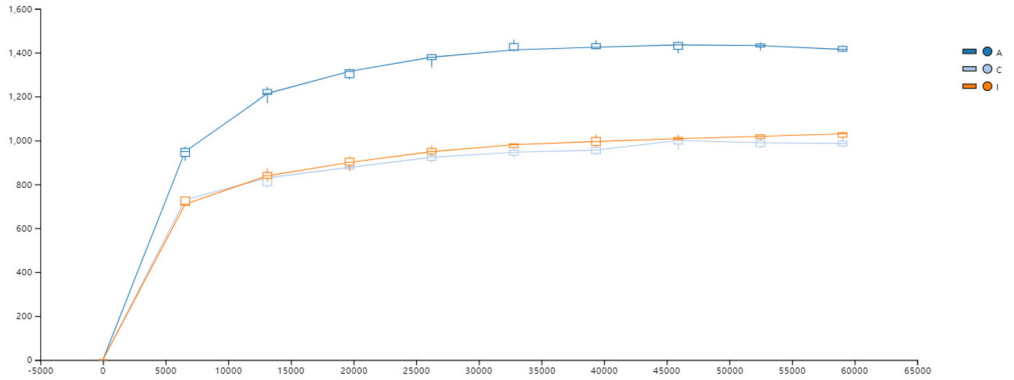

Fig. 5 Effect of LicA on rarefaction curve of T2DM mice. A: control group; C: LicA group (35 mg kg^{-1}); I: model group.

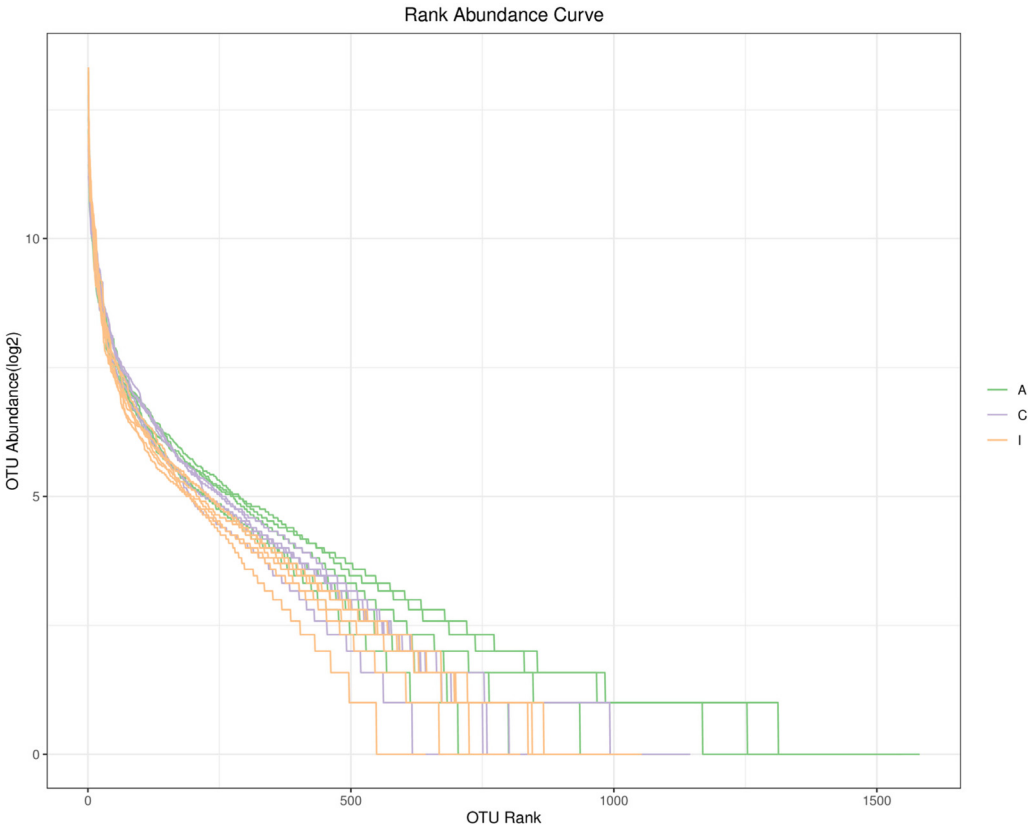

Fig. 6 The effect of LicA on the abundance distribution curve of T2DM mice. A: control group; C: LicA group (35 mg kg^{-1}); I: model group.

UPGMA cluster tree analysis

The UPGMA cluster tree analysis represents β diversity. Through the clustering analysis of all experimental samples, a sample-clustering tree can be constructed to study the similarity between different samples.[41] As shown in Fig. 8, the model group was divided into a large cluster, which was different from other groups. The effect of the LicA group on intestinal microbes in T2DM mice was significant, which was far away from the model group. The sample cluster of the LicA administration group was closer to that of the control group, indicating that LicA could change the composition of intestinal microbes in T2DM mice.

Venn diagram analysis of species. Venn diagrams can be used to count the number of common and unique species in multiple groups or samples, and can intuitively show the similarity and overlap of composition of species in different environmental samples. As shown in Fig. 9, the Venn chart analysis was represented by OTU samples with a similar level of 97%. The number of bacterial species OTU shared by the

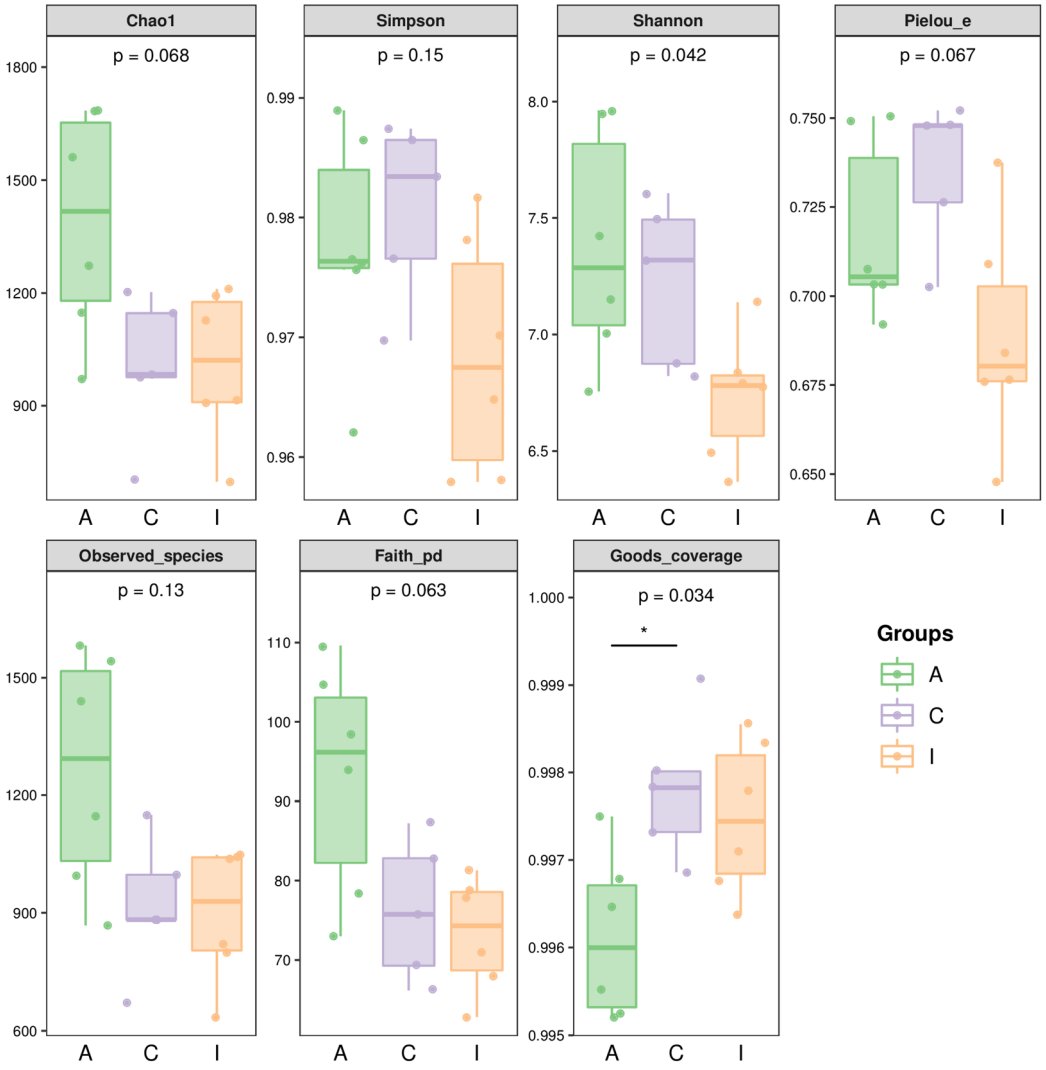

Fig. 7 Effects of LicA on alpha diversity index in T2DM mice. A: control group; C: LicA group (35 mg kg^{-1}); I: model group.

three groups of the control group, the model group, and the LicA group was 822. The number of bacterial species OTU shared by the LicA group and the control group was 551, which was higher than the number of bacterial species OTU shared by the LicA group and model group was 177. The results showed that the bacterial composition of the LicA group was similar to that of the control group. The results showed that the prevention and supplementation of LicA could improve the imbalance of intestinal flora in T2DM mice.

PCoA, PCA, and NMDS. The analysis of PCoA, NMDS, and PCA was used to evaluate the different levels of the intestinal flora among three samples (the control, model, and LicA groups). As shown in Fig. 10, the sample points of mice in the control group and the LicA group were distributed together, indicating that they have high similarity in intestinal flora diversity. The sample points of mice in the model group were far away from those in other groups, indicating that the diversity of intestinal flora in the model group was less similar to that in other groups. The above results indicate that LicA could improve the composition of species disorder of intestinal flora in T2DM mice.

Effect of LicA on the species composition of intestinal flora in T2DM mice

Based on the OTU results, the OTU was annotated with species, and the flora structure was studied from the phyla and genus levels to analyze the specific changes in intestinal flora. As shown in Fig. 11, the abundance of Firmicutes and Bacteroides was the highest and they were the most important phyla in feces. Compared with the control group, the F/B ratio of the model group was decreased ($p < 0.01$), while the F/B ratio of the LicA group was increased compared with the model group.

At the genus level, LicA administration can improve the relative abundance of some intestinal flora, including increasing the relative abundance of genera such as *Oscillospira*, *Ruminococcus*, *Helicobacter*, *Coprococcus*, *Rikenella*, and *Dehalobacteria*, and reducing the relative abundance of

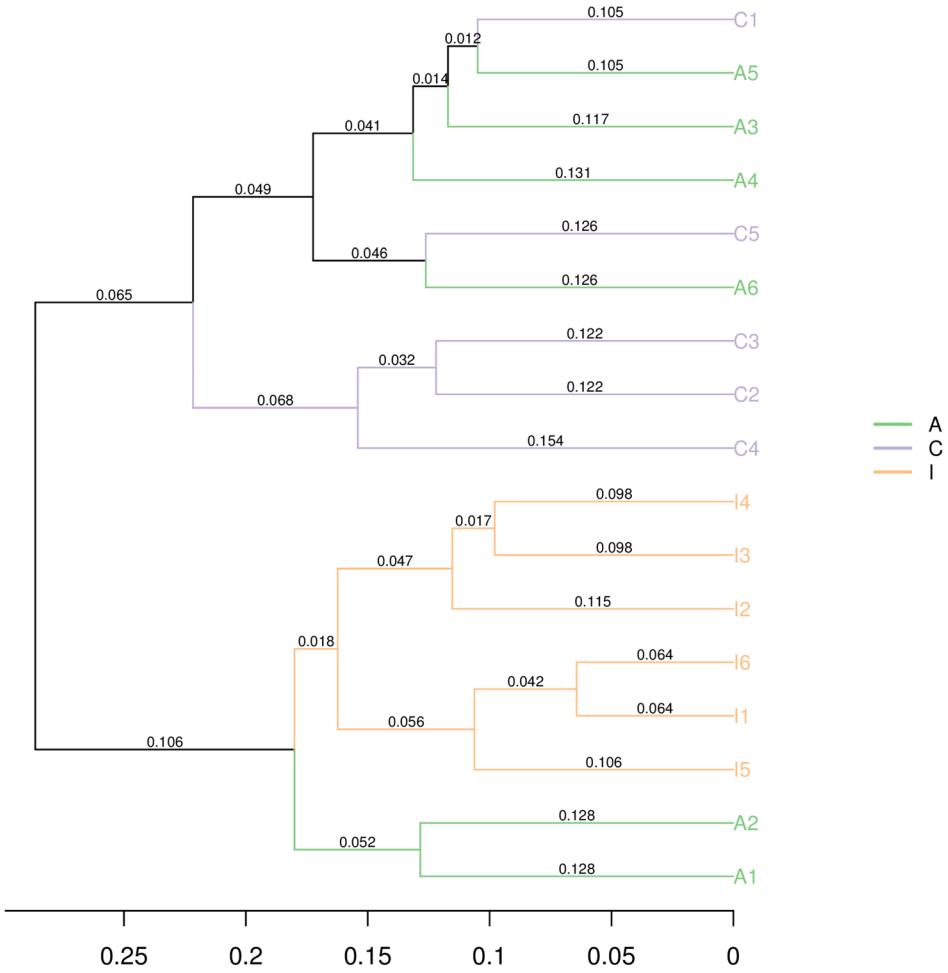

Fig. 8 Effect of LicA on hierarchical clustering analysis in type 2 diabetic mice. A: control group; C: LicA group (35 mg kg^{-1}); I: model group.

Lactobacillus. *Oscillospira*, *Helicobacter*, *Ruminococcus* and *Dehalobacterium* were the key genera with high abundance in the control and LicA groups.

At the level of the family, the top-ranked species were *Ruminococcaceae*, *Lachnospiraceae*, *S24-7*, *Rikenellaceae*, *Prevotellaceae*, *Helicobacteraceae*, *Desulfovibrionaceae*, *Lactobacillaceea*, and *Bacteroidaceae*. Compared with the control group, the abundance of *Ruminococcaceae* and *Lachnospiraceae* in the model group was decreased ($p < 0.05$), while the abundance of *S24-7* was increased ($p < 0.01$). In the LicA group, the abundance of *Ruminococcaceae* was significantly increased ($p < 0.05$), *Lachnospiraceae* was increased, and *S24-7* was obviously decreased ($p < 0.05$) compared with the model group.

At the level of class, the top-ranked species were *Clostridia*, *Bacteroidia*, *Epsilonproteobacteria*, *Deltaproteobacteria*, and *Bacilli*. The *Bacteroidia* of the model group was increased ($p < 0.01$) and *Clostridia* was decreased ($p < 0.05$) compared with the control group. Compared with the model group, *Bacteroidia* in the LicA group was significantly lowered ($p < 0.05$), while *Clostridia* was evidently increased ($p < 0.05$). At the level of the order, the top species were *Clostridiales*, *Bacteroidales*, *Desulfovibrionales*, and *Lactobacillales*. Compared with the control group, *Bacteroidales* in the model group were significantly increased ($p < 0.01$) and *Clostridiales* was significantly decreased ($p < 0.05$). Compared with the model group, *Bacteroidales* were evidently decreased ($p < 0.05$) and *Clostridiales* were significantly increased ($p < 0.05$) in the LicA group.

Correlation analysis of intestinal flora and phenotype of T2DM

The Spearman correlation was used to analyze the correlation between glucolipid metabolism parameters and intestinal flora in each group. As shown in Fig. 12, there was a significant difference in the correlation between the distribution of gut microbiota in the mice and the parameters of glucose and lipid metabolism. At the phylum level, *Firmicutes* was positively correlated with body weight and HDL-C levels ($p < 0.05$); *Firmicutes* was negatively correlated with FBG, OGTT, TC, and TG levels ($p < 0.05$). *Bacteroidetes* were positively correlated with levels of OGTT, HOMA-IR, LDL-C, TC, TG, and FBG ($p < 0.05$). At the genus level, *Helicobacter* was positively correlated with body weight and HDL-C levels ($p < 0.05$); *Helicobacter* was negatively correlated with FBG, OGTT, TC, TG, HOMA-IR, and

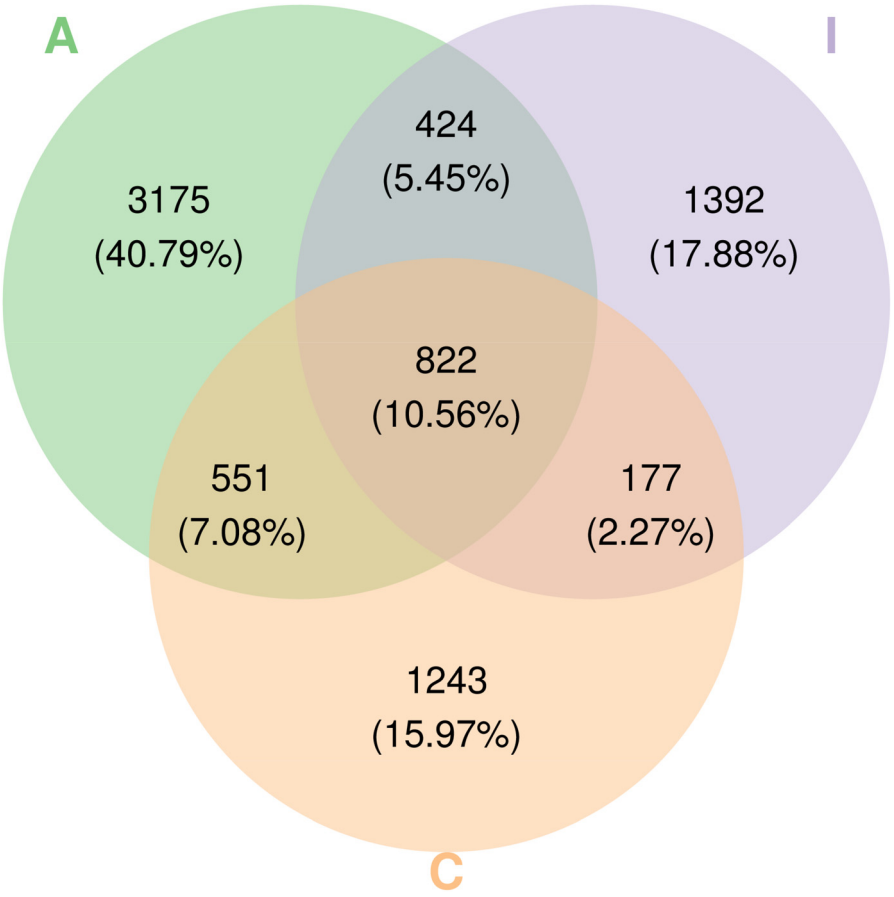

Fig. 9 Effect of LicA on Venn diagram of species in T2DM mice. A: control group; C: LicA group (35 mg kg^{-1}); I: model group.

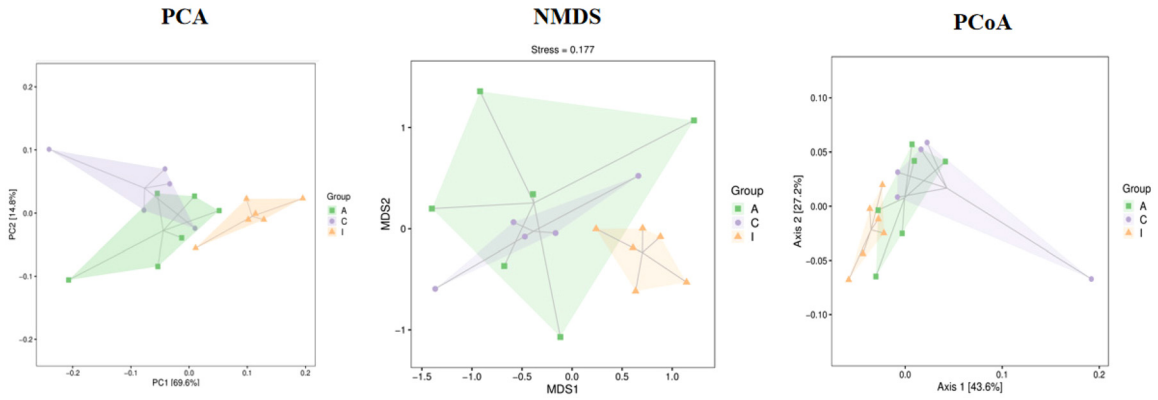

Fig. 10 PCA, NMDS, and PCoA plots rely on weighted UniFrac. A: control group; C: LicA group (35 mg kg^{-1}); I: model group.

LDL-C levels ($p < 0.05$). At the family level, *S24-7* was significantly correlated with FBG, OGTT, TC, TG, HOMA-IR, and LDL-C ($p < 0.05$); *Lachnospiraceae* and *Helicobacteraceae* were significantly correlated with body weight, insulin, and HDL-C ($p < 0.05$). At the class level, *Clostridia* was also evidently correlated with body weight, insulin, and HDL-C ($p < 0.05$); the correlation between *Bacteroidia* and FBG, OGTT, TC, TG, HOMA-IR, and LDL-C levels was significant ($p < 0.05$). At the level of order, *Clostridiales* and *Bacteroidales* were obviously correlated with the levels of FBG, body weight, insulin, OGTT, HOMA-IR, TC, TG, HDL-C, and LDL-C ($p < 0.05$).

Species heatmap analysis

Heatmaps are typically clustered based on similarities in abundance between species or samples. The species with high abundance and low abundance can be clustered in blocks, and

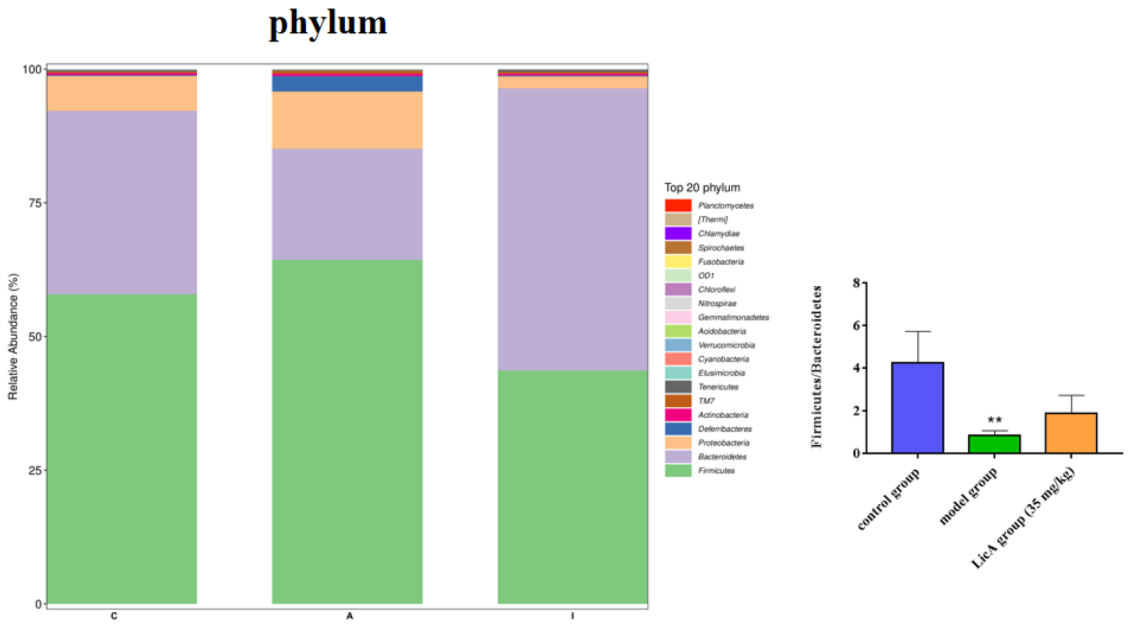

Fig. 11 Effects of LicA on the phylum, genus, family, class and order levels of taxonomic composition analysis in T2DM mice. A: control group; C: LicA group (35 mg kg^{-1}); I: model group. Data are expressed as the mean ± standard deviation (SD) (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control group. $^{\#}p < 0.05$ compared with the model group.

the similarity and difference in community composition of different groups at different classification levels can be reflected by a color change and similarity degree. It can be seen from the following genus classification heatmap (Fig. 13) that the abundance of the LicA group was similar to that of the control group, indicating that the species composition abundance between the two groups was similar. The abundance was different from that of the model group, indicating that the species composition and abundance were significantly different from those of the model group.

Species difference analysis

As shown in Fig. 14 and 15, LEfSe analysis was used to find the marker species of the mice with different treatments in this experiment. It was found that the marker bacteria of the control group were *Oscillospira*, *Alistipes*, *Dehalobacteria*, and *Candidatus_ Arthromitus*. The marker bacteria genera in the model group were *Anaerofustis*, *p_75_a5*, *Akkermansia*, *Allobaculum*, and *Sutterella*. The marker bacteria of the LicA group were *Anaerotruncus*, *Mucispirillum*, *Desulfovibrio*, *Coprobacillus*, *Jeotgalicoccus*, *Bilophila*, *Rikenella*, *Sphingomonas*, *Oligella*, *Facklamia*, *Corynebacterium*, *Psychrobacter*, and *Aerococcus*.

Discussion

The intestinal flora consists of large and complex microbial communities in the intestine, an important "microbial organ". The flora itself (or its metabolites) can affect many biological functions of the host. An imbalance of intestinal flora can lead to a series of complications, such as decreased immunity and an imbalance of energy metabolism, which may further lead to metabolic disorders, insulin resistance,[42] and, eventually, T2DM. T2DM is a metabolic disorder characterized by hyperglycemia caused by insulin resistance and impaired glucose tolerance.[43]

Many studies have confirmed the correlation between disorders of the intestinal flora and T2DM.[44,45] Furthermore, because the intestinal flora has a feedback regulation effect on diabetes,[46,47] regulating and improving intestinal flora may be a potential target for the prevention and treatment of T2DM. Gene abundance of human intestinal flora is closely related to lipid metabolism and insulin resistance.[42] People with a low genetic abundance of intestinal flora are more likely to have lipid metabolism disorders and insulin resistance than those with a high gene abundance.[48,49] Therefore, people with a low abundance of intestinal flora have a higher risk of developing prediabetes and T2DM.

The goal of this study, which was the first to use 16S rRNA sequencing to analyze changes in intestinal flora among three groups of samples, was to understand the effects of LicA on the intestinal flora of diabetic mice. In this animal experiment, mice in the model group were fed an HFD diet and injected with STZ to induce the T2DM model. We found that, compared with the model group, LicA helped control higher FBG levels in T2DM mice. In addition, LicA intervention also reduced the degree of IR and lipid disorders in T2DM mice. Studies have shown that when the abundance of intestinal flora is low, the content of TG increases.[50] Therefore, decreases in the TG levels of mice in the LicA intervention group may be related to increased intestinal flora abundance. The results of

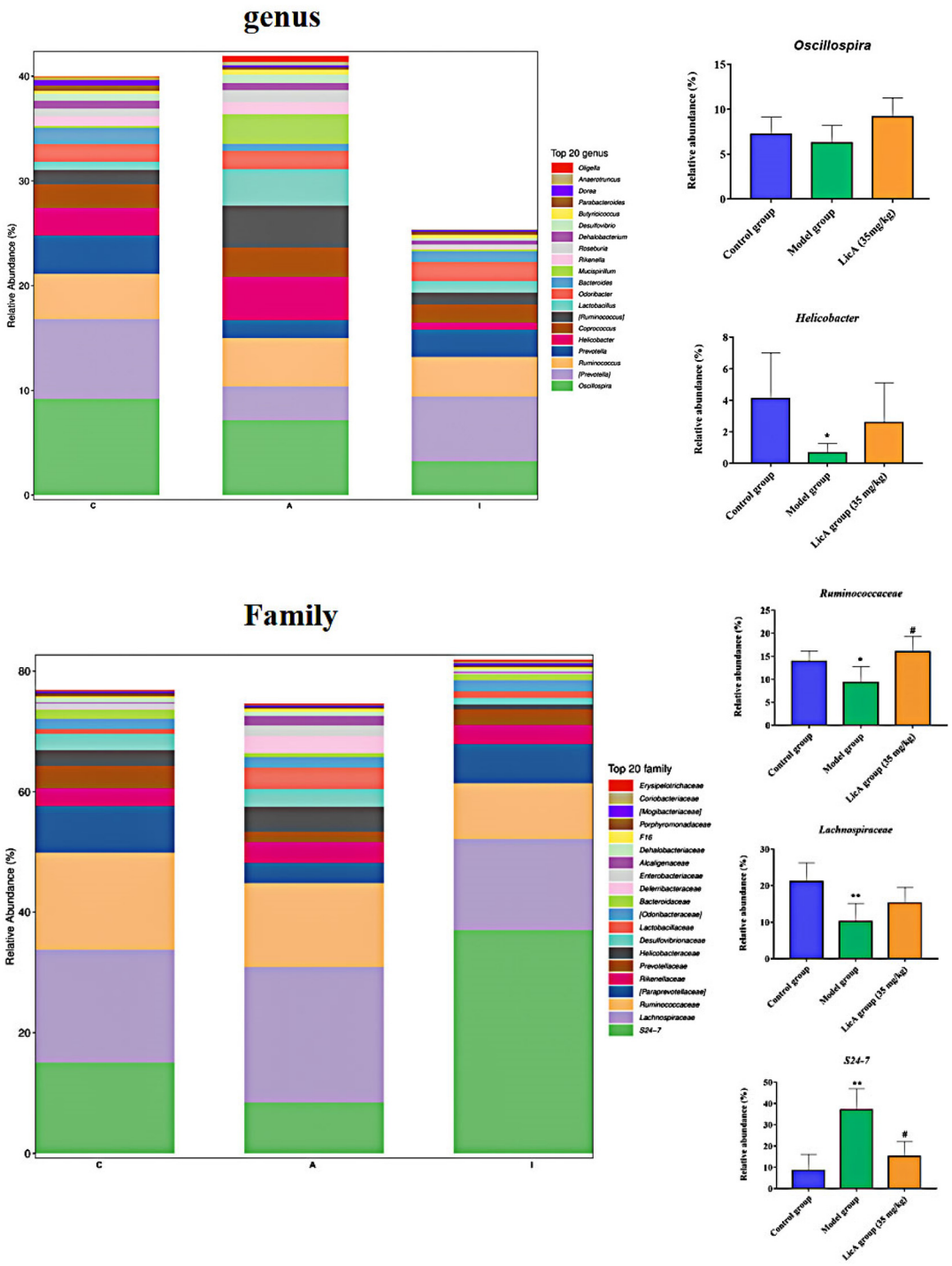

Fig. 11 *(Contd).*

Spearman's correlation analysis confirmed that intestinal flora was involved in the regulation of TG. HE staining also confirmed that liver lipid droplets in the LicA intervention group showed a decreasing trend compared with the model group.

At the same time, LicA reshaped the intestinal microbiota in T2DM mice. The dilution curve of this sequencing was flat, indicating that the sequencing sample size was sufficiently large. The diversity index can be used to evaluate the overall change in intestinal flora species.[47] All four indexes of alpha diversity in the T2DM model mice decreased, indicating a decrease in the richness, diversity, and evenness of intestinal microflora in the model mice, which is consistent with the experimental conclusions of Yuan.[51] LicA increased the four indexes of alpha diversity, indicating that it might improve the

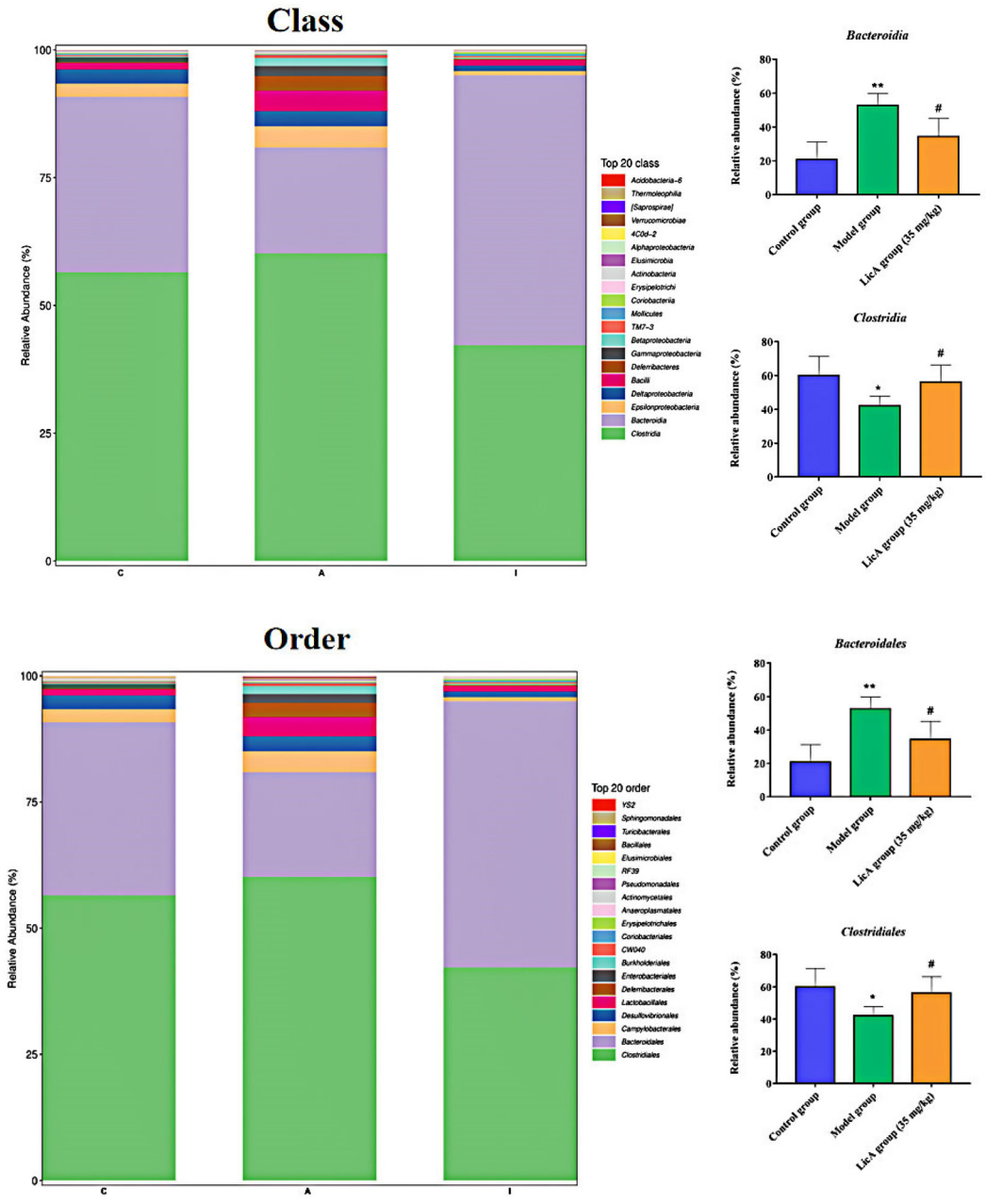

Fig. 11 *(Contd)*.

richness, diversity, and uniformity of intestinal flora in T2DM mice. Furthermore, UPGMA showed that the LicA and control groups clustered together. In contrast, the model group was clustered separately, which suggests that LicA improves the intestinal bacterial imbalance induced by HFD and STZ.

When IR occurs in T2DM, the body will produce more insulin in compensation, thus causing hyperinsulinemia. In this batch of animal experimental results, the serum insulin content of mice in the T2DM model group showed a decreasing trend, which is inconsistent with many reported results.[52,53] However, Msomia *et al.* also observed this phenomenon.[54] The reason for this phenomenon may involve the dysfunction of mouse islet cells induced by HFD and STZ or the functional damage of islet cells caused by the long-term compensatory IR state. In addition, it may be related to an increase in the secretion of other related factors, resulting in a decrease in the release of fasting insulin.

According to relevant literature, the composition of intestinal flora is significantly different in patients with T2DM compared with healthy people.[44,45,47,50] Compared with the control mice, the level of *Bacteroidetes* in the T2DM model mice increased at the phylum level. In contrast, the level of *Firmicutes* decreased. That is, the *Firmicutes*/*Bacteroidetes* ratio decreased in the T2DM model group. In contrast, in the LicA

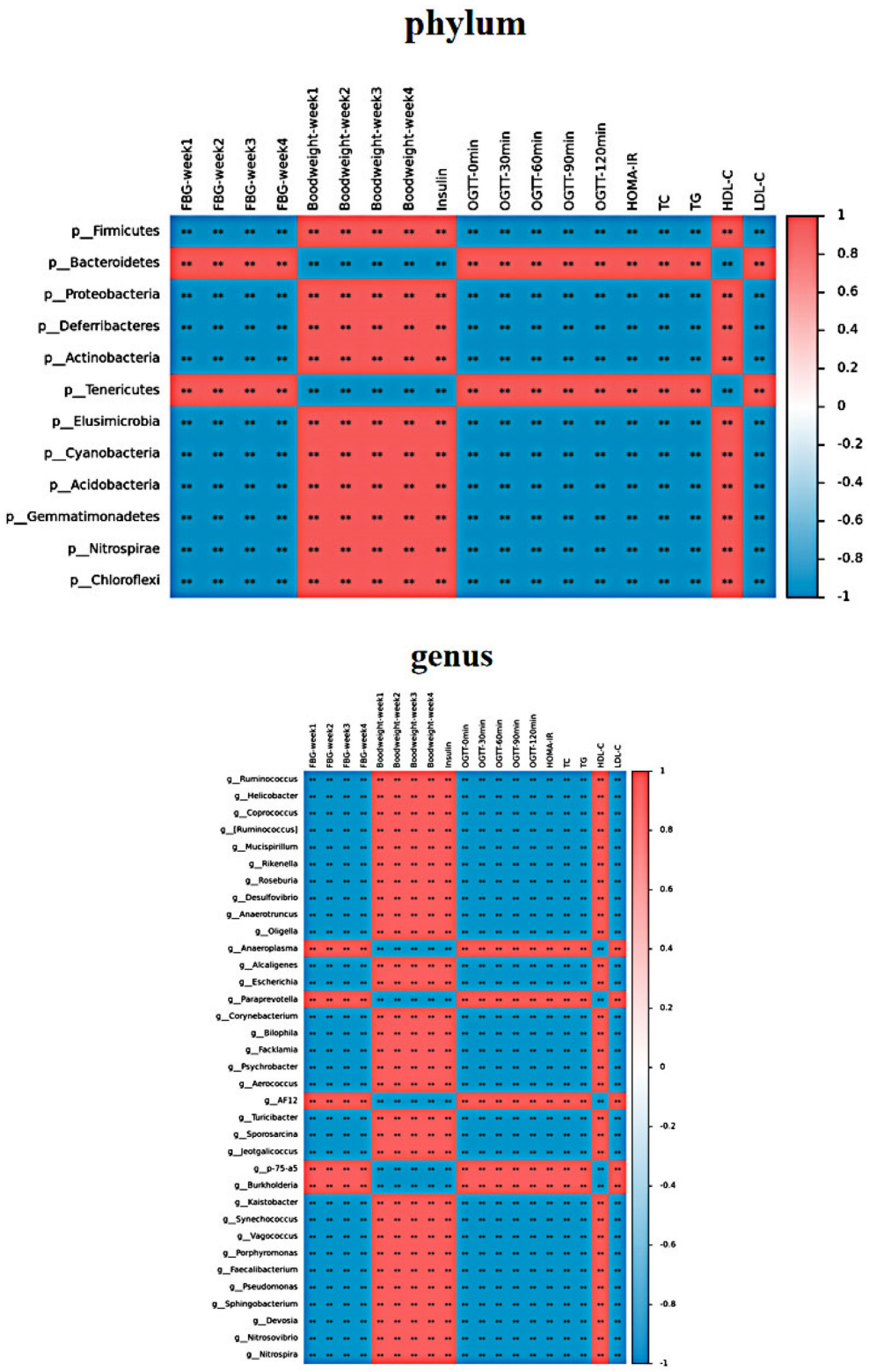

Fig. 12 Cluster thermogram of Spearman correlation analysis results, the correlations between glucose and lipid metabolism-related parameters and intestinal flora at phylum, class, order, family, and genus level.

group, the abundance of *Bacteroides* decreased, and the abundance of *Firmicutes* increased, resulting in an increased *Firmicutes*/*Bacteroidetes* ratio. Other studies have documented similar changes in intestinal flora in T2DM.[44,45,51] In terms of genus classification, *Oscillospira*, *Prevotella*, *Ruminococcus*, *Helicobacter*, *Coprococcus*, *Rikenella*, *Dehalobacterium*, and

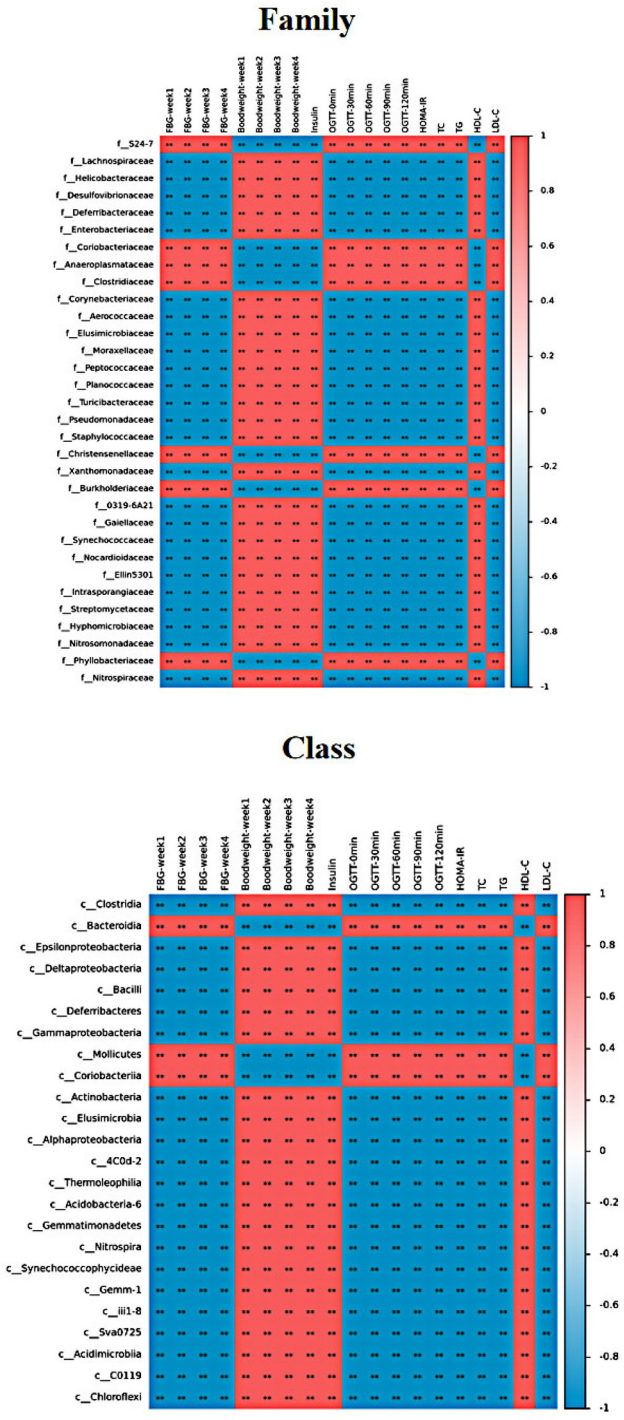

Fig. 12 *(Contd)*.

Dorea in the LicA group were higher than those in the model group. Studies have shown that *Oscillospira* and *Rikenella* are involved in the improvement of blood sugar and lipid metabolism.[55,56] *Prevotella* is one of the essential microbes found in the human oral cavity and large intestine. The symbiotic *Prevotella* of the intestinal tract contributes to the decomposition of polysaccharides, converts excess polysaccharides into short-chain fatty acids, promotes the absorption

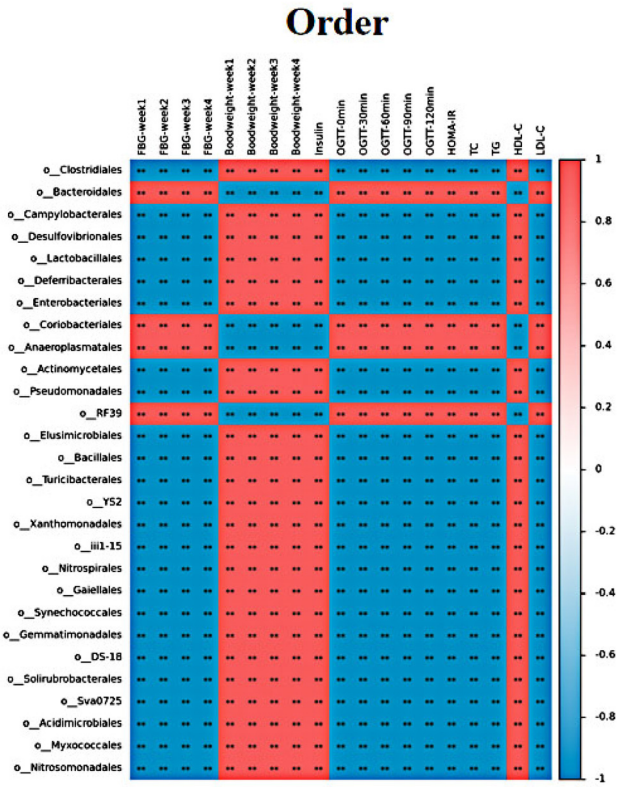

Fig. 12 *(Contd)*.

of free fatty acids and glucose, and improves IR status.[46,57] In recent years, metagenomics research has found that the composition of intestinal flora in healthy people can be divided into the following three dominant groups: *Ruminococcus*, *Prevotella*, and *Bacteroidetes*.[52–56] The decrease in the abundance of *Ruminococcus* is affected by the normal process of glucose and lipid metabolism. *Coprococcus* is involved in the butyric acid production pathway, including the phosphate/butyrate kinase pathway and the butyryl CoA/acetyl CoA transfer pathway.[58] *Helicobacter* species induce regulatory T cells and T follicular helper cells under homeostatic conditions.[59] Chen *et al.* also reported that *Ganoderma lucidum* polysaccharide treatment increased the level of *Dehalobacterium*.[60] *Dorea* is directly related to glucose metabolism.[61]

Regarding family, class, and order classifications, *Ruminococcaceae*, *Lachnospiraceae*, *Clostridia*, and *Clostridiales* in the LicA group were higher than those in the model group. *Lachnospiraceae* and *Ruminococcaceae* are short-chain fatty acid-producing bacteria.[62] Studies have shown that serum-conjugated C-6 hydroxylated bile acids are associated with human metabolic health and the intestinal community of *Clostridium* species.[63] In the LicA group, *S24-7*, *Bacteroidia*, and *Bacteroidales* were less abundant than those in the model group. Studies have shown that *Bacteroides*, and *S24-7* are the dominant bacteria related to T2DM.[64]

The correlation analysis showed that *Firmicutes* and *Helicobacter* were positively correlated with body weight and

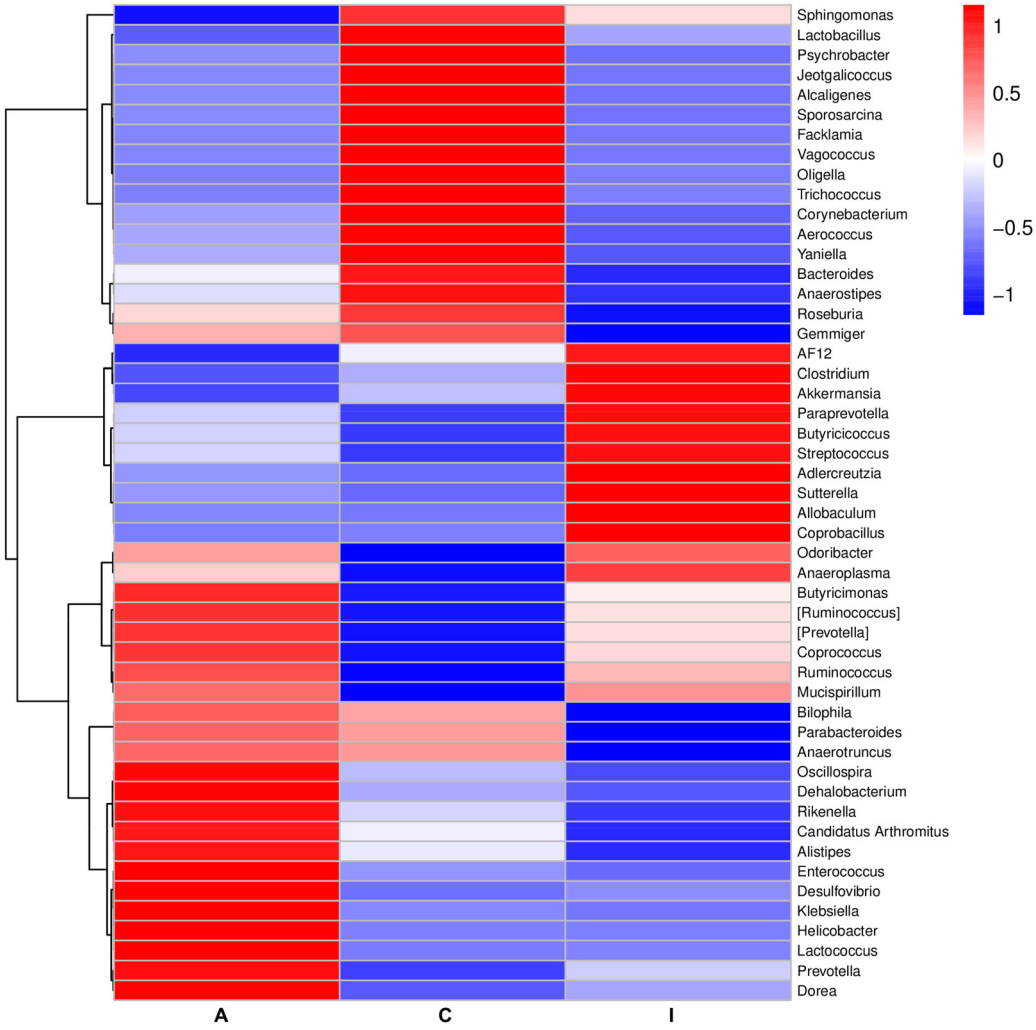

Fig. 13 Effect of LicA on intestinal flora structure of T2DM mice. A: control group; C: LicA group (35 mg kg^{-1}); I: model group.

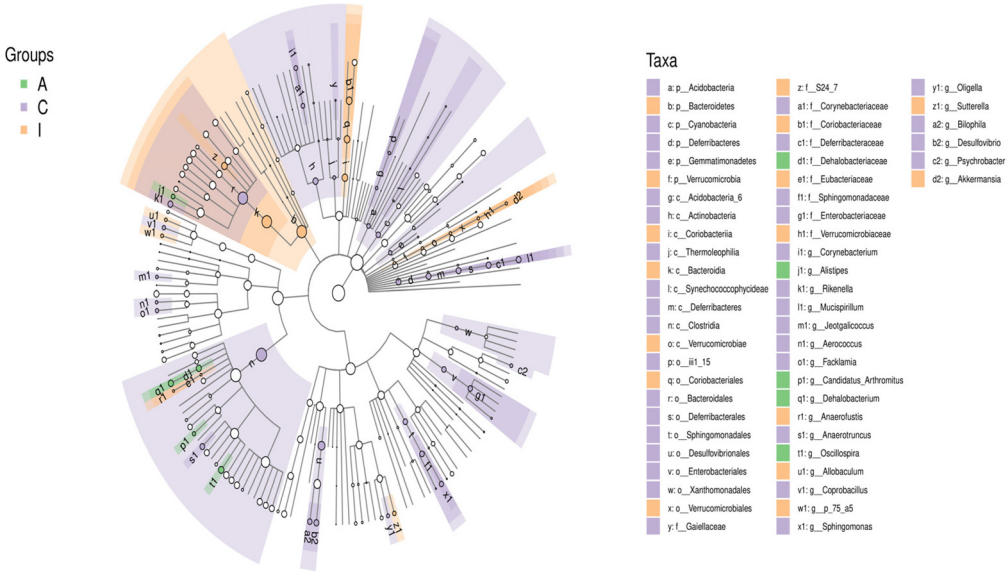

Fig. 14 Effect of LicA on cladogram of T2DM mice. A: control group; C: LicA group (35 mg kg^{-1}); I: model group.

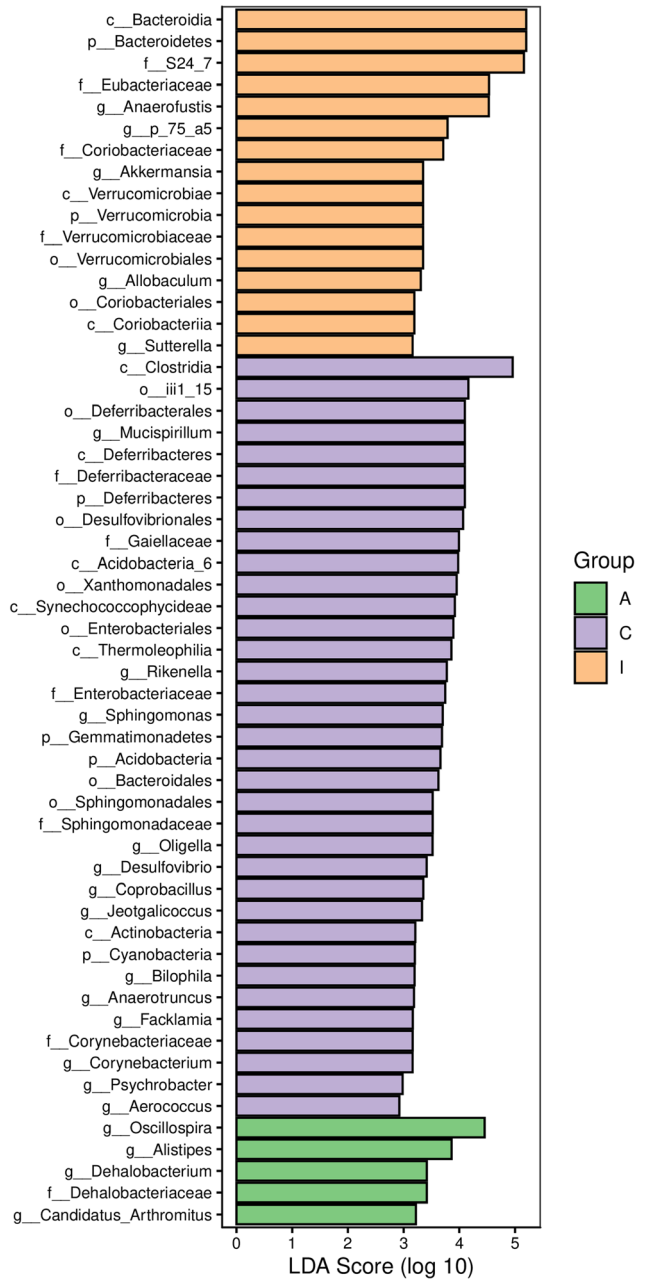

Fig. 15 Effect of LicA on the LDA value distribution of T2DM mice. A: control group; C: LicA group (35 mg kg^{-1}); I: model group.

HDL-C levels. *Firmicutes* and *Helicobacter* were negatively correlated with FBG, OGTT, TC, and TG levels. In contrast, the abundance of *Bacteroidetes* was positively correlated with levels of OGTT, LDL-C, TC, TG, and FBG. Furthermore, *S24-7*, *Lachnospiraceae*, *Helicobacteraceae*, *Clostridia*, *Bacteroidia*, *Clostridiales*, and *Bacteroidales* were significantly correlated with FBG, body weight, insulin, OGTT, HOMA-IR, TC, TG, HDL-C, and LDL-C levels. Wan *et al.* reported that Ruminococcus may play an important role in the treatment of diabetes.[65,66] This study also demonstrated that the intestinal flora abundance of *Ruminococcus* was negatively correlated with blood glucose, IR, LDL-C, and TG. However, it was positively correlated with HDL-C and FINS. The results of this experiment suggest that LicA treatment might increase the level of *Ruminococcus*.

Meanwhile, the Venn diagram of intestinal flora showed that the composition of intestinal flora in the licA group differed from that in the T2DM model group. These observations suggest that LicA had an antidiabetic effect by altering the *Firmicutes*/*Bacteroides* ratio and regulating the level of other specific bacterial species. In this study, the types of pathogenic bacteria in the stools of mice with T2DM differed from those reported in the literature.[45–47,67] These differences may be explained by different subjects, experimental environments, and diet structures.

LEfSe analysis was used to compare the variance between groups at the relative abundance level. It showed significant differences in 56 bacterial branches among the three groups. In the histogram of a linear discriminant analysis (LDA) score, biomarkers with substantial differences between different groups are marked, and changes in flora at the genus level are of great significance for the study of flora-host interactions. At the end of the experiment, the analysis of different species among groups showed that changes in bacterial levels (for *Anaerotruncus*, *Mucispirillum*, *Desulfovibrio*, *Coprobacillus*, *Jeotgalicoccus*, *Bilophila*, *Rikenella*, *Sphingomonas*, *Oligella*, *Facklamia*, *Corynebacterium*, *Psychrobacter*, and *Aerococcus*) might be related to the therapeutic effect of LicA on diabetes. In addition, some rare bacteria were detected, such as *Psychrobacter* and *Musicillum*. However, the impact of these bacteria on health is still unclear, and further animal research will be needed to decipher their complex and multimodal disease mechanisms.

Conclusions

In conclusion, LicA improved the symptoms of hyperglycemia and intestinal flora disorders in the intestinal tracts of mice with T2DM. Correlation analysis results showed that intestinal flora was significantly correlated with the parameters of glycolipid metabolism. These results suggest that intestinal microbiota can affect lipid metabolism and FBG levels. It appears that LicA may have multiple pathways to reduce the IR status of T2DM mice and alleviate disorders of glycolipid metabolism induced by T2DM. In the presence of LicA, the composition of intestinal flora might be one of the key factors driving the host to resist diabetes. Moreover, this work provided clues to explore the potential factors that may improve the therapeutic effect of anti-diabetes agents. Further research on personalized therapeutic strategies based on intestinal microbiota will help enhance the efficacy of anti-T2DM agents in mice.

Author contributions

Zhonghua Luo: methodology, writing – original draft, writing – review and editing, experimental operation, visualization. Jing

Xu: resources, supervision, formal analysis. Qingqing Gao and Zhifang Wang: Conceptualization, experimental operation, methodology, and experimental operation. Yunen Liu: Project administration. Mingxiao Hou: Funding acquisition.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgements

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