

# 16S rRNA gene sequencing analysis of gut microbiome in a mini-pig diabetes model

Miaomiao Niu | Yuqiong Zhao | Lei Xiang | Yunxiao Jia | Jifang Yuan | Xin Dai | Hua Chen 

Laboratory Animal Center, Chinese PLA General Hospital, Beijing, PR China

## Correspondence

Hua Chen, 3rd floor, East ward Building, No. 28 Fuxing Road, Haidian District, Beijing, PR China.  
Email: chenhua301paper@163.com

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## Abstract

**Background:** Currently, increasing attention is being paid to the important role of intestinal microbiome in diabetes. However, few studies have evaluated the characteristics of gut microbiome in diabetic miniature pigs, despite it being a good model animal for assessing diabetes.

**Methods:** In this study, a mini-pig diabetes model (DM) was established by 9-month high-fat diet (HFD) combined with low-dose streptozotocin, while the animals fed standard chow diet constituted the control group. 16S ribosomal RNA (rRNA) gene sequencing was performed to assess the characteristics of the intestinal microbiome in diabetic mini-pigs.

**Results:** The results showed that microbial structure in diabetic mini-pigs was altered, reflected by increases in levels of *Coprococcus\_3* and *Clostridium\_sensu\_stricto\_1*, which were positively correlated with diabetes, and decreases in levels of the bacteria *Rikenellaceae*, *Clostridiales\_vadinBB60\_group*, and *Bacteroidales\_RF16\_group*, which were inversely correlated with blood glucose and insulin resistance. Moreover, PICRUSt-predicted pathways related to the glycolysis and Entner-Doudoroff superpathway, enterobactin biosynthesis, and the L-tryptophan biosynthesis were significantly elevated in the DM group.

**Conclusion:** These results reveal the composition and predictive functions of the intestinal microbiome in the mini-pig diabetes model, further verifying the relationship between HFD, gut microbiome, and diabetes, and providing novel insights into the application of the mini-pig diabetes model in gut microbiome research.

## KEYWORDS

16S rRNA, diabetes, gut microbiome, mini-pig

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## 1 | INTRODUCTION

Diabetes, a disease characterized by abnormal metabolism, is becoming increasingly prevalent around the world, in large part because of growing use of refined sugar and fats, oils, and meats.<sup>1–3</sup> In the past 20 years, rodent animals have been the most widely used animal model to study diabetes. This extensive usage can be explained by their small size, efficient and specific genetic modifications, and relatively inexpensive experimental cost.<sup>4</sup> Recently, miniature pigs have become increasingly popular in diabetes research, owing to their similarities to humans in terms of anatomy, physiology, pharmacokinetics, pancreas architecture, and insulin's molecular structure and function, as well as the possibility of dietary and surgical interventions.<sup>5,6</sup> High-fat diet (HFD) with a subsequent injection of low-dose streptozotocin (STZ) is considered to be a classical and effective approach, and represents a good model to mimic the development of diabetes from glucose intolerance to insulin resistance and, finally, partial  $\beta$ -cell death induced by STZ.<sup>7</sup>

Nowadays, accumulating evidence has revealed the critical role of the intestinal microbiome in the development of diabetes.<sup>8–11</sup> Gut microbial dysbiosis can lead to metabolic endotoxemia, intestinal hyperpermeability, and low-grade inflammation, which are closely related to diabetes.<sup>12,13</sup> Mice have been used frequently in gut-microbiota-related research, though there are some gene-level differences in gut metagenomes between mouse and human.<sup>14</sup> Therefore, because their gut composition is highly similar to that of humans,<sup>15–17</sup> mini-pigs may be a potential animal model for gut-microbiota-related studies. In this study, we evaluated the composition and predictive functions in a mini-pig diabetes model established by a 9-month HFD with low-dose STZ, to explore its potential application in gut microbiota research.

## 2 | METHODS

### 2.1 | Ethical statement

All experiments involving animals had approval from the Institutional Animal Care and Use Committee of Chinese PLA General Hospital, and were conducted under the committee's guidelines (ID: 2018-D14-26).

### 2.2 | Animal experiments

Eight 6-month-old Bama mini-pigs (12–15 kg) were purchased from the Beijing Shi Chuang Century mini-pig breeding base, and housed in a conventional environment at a temperature of 20–26°C and 40%–70% relative humidity, under a 12 h/12 h light-dark cycle. Mini-pigs were maintained in single cages and drank water freely, for 1 week for adaptation. Then, the 8 Bama mini-pigs were randomized to the chow and DM groups. Pigs in the chow group were fed a standard diet containing 23.4 kcal% protein, 66.0 kcal% carbohydrate, and

10.6 kcal% fat. The DM group was fed a HFD containing 17.7 kcal% protein, 38.7 kcal% carbohydrate, and 43.6 kcal% fat, and underwent intraperitoneal injection with low-dose STZ (90 mg/kg) 3 months later. All the animals were fed at 3% body weight, with adjustment according to body weight change every month. After 9 months of treatment, each pig was anesthetized individually and underwent immediate dissection. The luminal content of the distal colon was obtained within 30 min post-euthanasia, snap frozen in liquid nitrogen and kept at –80°C.

### 2.3 | Assessment of clinical characteristics

Body weight and blood lipid indexes, including triglyceride, total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL), fasting blood glucose, and fasting insulin amounts were measured each month. HOMA-IR (fasting plasma glucose  $\times$  fasting plasma insulin/22.5) was used as an insulin resistance index. Moreover, intravenous glucose tolerance test (IVGTT) was performed every 3 months as previously reported to evaluate STZ-HFD's effect on glucose tolerance in animals.<sup>18</sup>

### 2.4 | 16S rRNA gene sequencing

For DNA extraction, we utilized the E.Z.N.A. Stool DNA Kit (D4015; Omega, USA) as directed by the manufacturer. 16S rRNA gene sequencing was carried out by Lian Chuan Bio Technology Company Limited (China). Microbial 16S V3–V4 underwent amplification with modified 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers.<sup>19,20</sup> Polymerase chain reaction (PCR) products were assessed by 2% agarose gel electrophoresis, purified with AMPure XT beads (Beckman Coulter Genomics, USA) and quantitated with Qubit (Invitrogen, USA). Then, amplicons were pooled for sequencing; the amplicon library was evaluated on an Agilent 2100 Bioanalyzer (Agilent, USA) using the Library Quantification Kit for Illumina (Kapa Biosciences, USA). PhiX Control library (v3) (Illumina) was mixed with the obtained amplicon library (expected at 30%). Library sequencing was carried out by 2 300PE MiSeq runs, utilizing standard Illumina sequencing primers.

### 2.5 | Sequence data analysis

Sequencing was carried out on an Illumina MiSeq platform (LC-Bio) as directed by the manufacturer. Paired-end reads were attributed to specimens according to unique barcodes, and truncated via removal of barcodes and primer sequences. FLASH was applied for paired-end read merging. The fqtrim software (V0.94) was utilized for filtering. Chimeric sequences were removed with Vsearch v2.3.4. Sequences showing 97% similarity or higher were considered to indicate the same operational taxonomic unit (out) based on Vsearch. The Ribosomal Database Project (RDP) classifier was utilized to

assign taxonomic data to various representative sequences. Multiple sequence alignment was carried out with mafft V7.310 for assessing phylogenetic relationships among operational taxonomic units (OTUs). The least represented sequences were utilized to normalize OTU abundance. Alpha diversity was assessed using Chao1, Shannon, and Shannon indices, determined with QIIME 1.8.0, while beta diversity (species complexity) was assessed by principal coordinates analysis (PCoA) and cluster analysis with QIIME 1.8.0.

## 2.6 | Statistical analyses

Data are presented as mean  $\pm$  standard error of the mean (SEM). For group comparison analysis, *t*-test was carried out with SPSS 22.0 (SPSS, USA).  $p < .05$  indicated statistical significance.

## 3 | RESULTS

### 3.1 | Characteristics of DM mini-pigs

As shown in Figure 1, body weight, fasting blood glucose, blood lipid, HOMA-IR index, and glucose tolerance were comparable in the chow and DM groups before the treatment. During the study, the animals in the chow group were in good condition and had normal diet. In the first 3 months of HFD, body weight was higher in the DM group compared with the chow group (Figure 1A,  $p < .05$ ), while fasting blood glucose, blood lipid, HOMA-IR index, and glucose tolerance were similar in both groups. After STZ was administered to the DM group at the beginning of the 4th month, the animals showed listlessness and loss of appetite. Three days later, the state of animals in the DM group gradually returned to normal, but their weights increased slowly, and the body weights were comparable in both groups between the 4th and the 9th month. However, fasting blood glucose amounts, serum triglyceride, and HOMA-IR index of the DM group were remarkably elevated compared with the chow group after STZ application ( $p < .05$ ), and these clinical characteristics lasted until the end of the study (Figure 1A–E). IVGTT results indicated that glucose tolerance was also impaired in diabetic animals at the 6th and 9th month (Figure 1D). The average values of serum total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were within the normal range, and showed no difference (except total cholesterol at the 4th month) between the 2 groups during the experiment (Figure 1F–H). These results showed that we have obtained a miniature pig model of diabetes with hyperglycemia, high triglyceride, insulin resistance, and impaired glucose tolerance.

### 3.2 | Alterations in the intestinal microbiome after DM establishment

After 9 months of high-fat diet treatment, we assessed the gut microbiota structure of colon contents in the chow and DM groups via

sequencing of 16S rRNA's V3–V4. In total, 593,690 reads were detected in the 8 samples. Then, alpha diversity was assessed, and the Chao1 and Shannon indices were lower but showed no significant differences in the DM group compared with controls (Figure 2A,B). Meanwhile, Simpson index values were similar (Figure 2C). However, PCoA of weighted UniFrac distances based on OTUs indicated that the intestinal microbiome had remarkable structural changes after DM induction (Figure 2D, ANOSIM  $p = .026$ ).

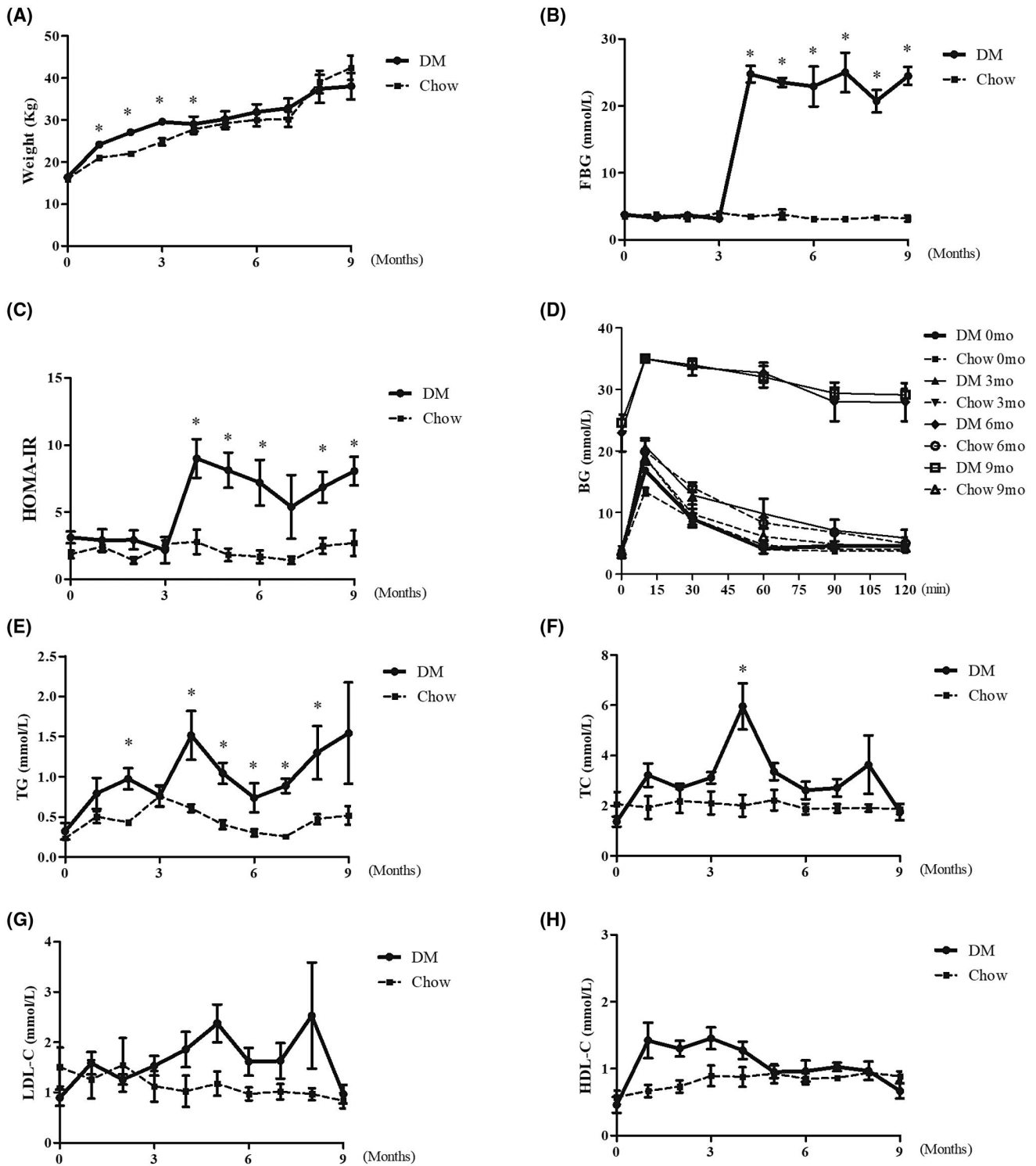
Bacterial composition at the taxonomic level was then investigated. Similar to human and rodent data,<sup>21,22</sup> the most represented bacterial phyla in all minipigs included Firmicutes (64.24%) and Bacteroidetes (24.20%), followed by Proteobacteria (6.79%), Spirochaetes (2.03%), and Actinobacteria (0.85%) (Figure 3A). Meanwhile, linear discriminant analysis (LDA) effect size (LEfSe) analysis was carried out for determining specific gut microbes that were differentially enriched in diabetic animals in comparison with controls. In total, 38 differential bacterial taxa were identified by a logarithmic LDA score threshold of 3.0. Several genera, including *Lactobacillus*, *Peptococcus*, *Coprococcus\_3*, *Clostridium\_sensu\_stricto\_1*, and *Paludibacter*, were enriched in the microbiota of the DM group. Meanwhile, *Rikenellaceae\_RC9\_gut\_group*, *Prevotella\_7*, and some unclassified genera of *p\_2534\_18B5\_gut\_group*, *Clostridiales\_vadinBB60\_group*, *Bacteroidales\_RF16\_group*, *Izimaplasmatales*, and *Peptococcaceae* were significantly underrepresented in the DM group (Figure 3B). The cladogram in Figure 3C reveals remarkable phylogenetic differences in the intestinal microbiome between the DM and chow groups.

### 3.3 | Correlation analysis of enriched genera in the chow and DM groups

The potential associations of diabetes with structural changes of the gut microbiome were assessed. As shown in Figure 4, networks comprising genera enriched in the DM group had less relevance compared with those of controls. In total, 24 microbiome entities showed significant associations, with  $|r| > .4$  and  $p < .05$  (Figure 4B). For instance, *Lactobacillus* was positively associated with *Rikenellaceae\_RC9\_gut\_group* and negatively correlated with *Paludibacter*. In addition, *Rikenellaceae\_RC9\_gut\_group* and *Paludibacter* were both negatively associated with *p-2534-18B5\_gut\_group\_unclassified*, and *Izimaplasmatales\_unclassified*. *Peptococcus* was positively associated with *Izimaplasmatales\_unclassified* and negatively correlated with *Clostridium\_sensu\_stricto\_13*.

### 3.4 | Functional changes of the intestinal microbiota in the DM group

To further assess the gut microbiota's functional properties, PICRUSt2 was utilized for predicting the functional composition of the colonic microbiota. PICRUSt2 pathway analysis showed 25 altered pathways in DM animals versus controls, including 11 that

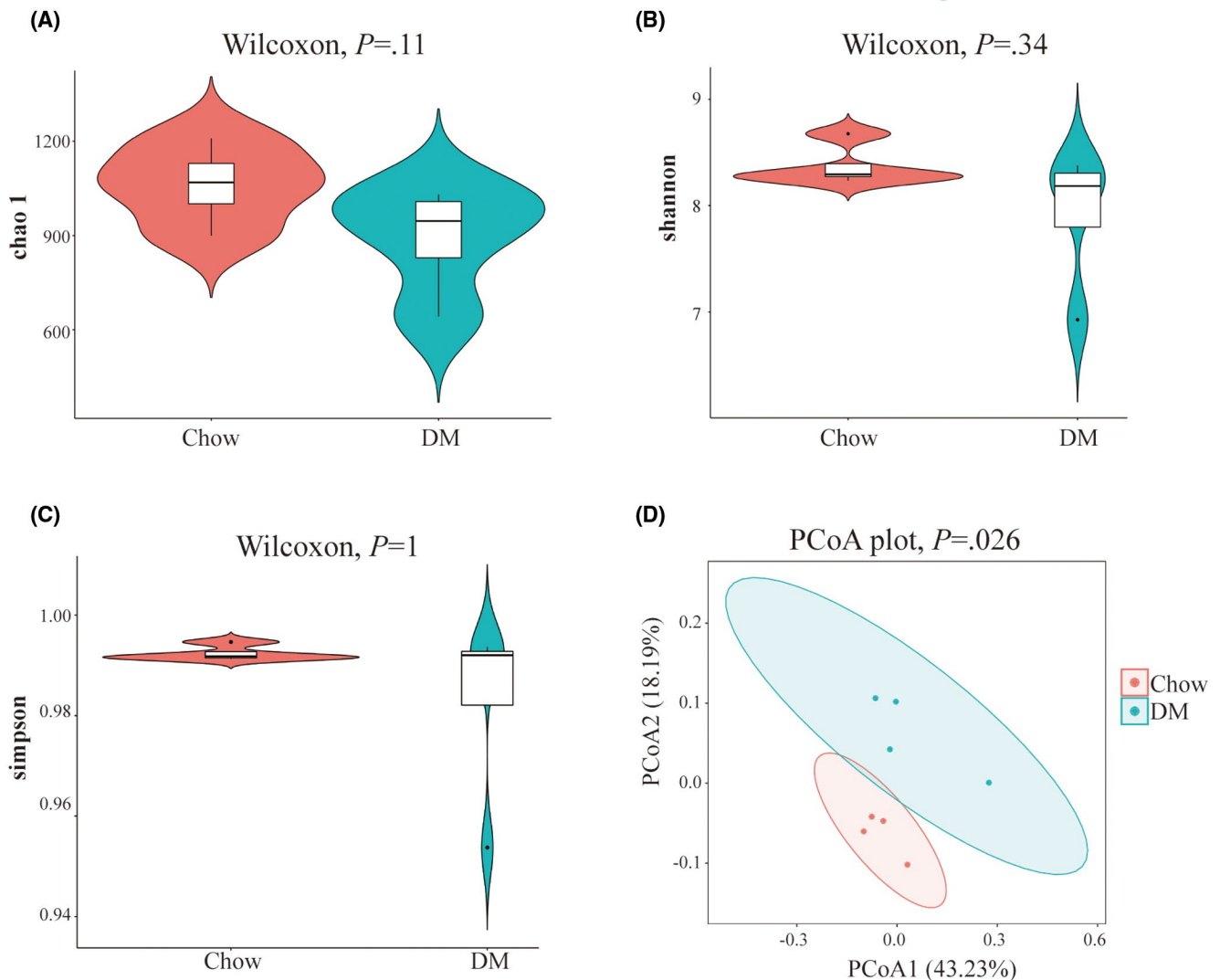


**FIGURE 1** Clinical characteristics of the chow and DM groups. (A) Body weight. (B) Fasting blood glucose amounts. (C) HOMA-IR amounts. (D) IVGTT results. Serum lipid levels were assessed, including (E) triglyceride, (F) total cholesterol, (G) LDL, and (H) HDL

were overrepresented in the DM group ( $p < .05$ ). Pathways associated with the glycolysis and Entner-Doudoroff superpathway, enterobactin biosynthesis, superpathway of L-tryptophan biosynthesis, superpathway of N-acetylneuraminate degradation, and mixed acid fermentation were significantly elevated in the DM group (Figure 5).

## 4 | DISCUSSION

Various reports indicate that gut microbiota imbalance has an important function in diabetes.<sup>8-10</sup> Rodent animals, especially mouse, are the most commonly used animal models. Firmicutes and Bacteroidetes are the top 2 phyla gut microbiota in both human



**FIGURE 2** Gut microbial diversity comparison between the chow and DM groups. Alpha diversity was assessed via the (A) Chao 1, (B) Shannon, and (C) Simpson indexes of OTU proportions. Principal coordinates analysis of beta diversity utilized (D) weighted UniFrac to analyze OTU levels

and mice; however, some bacterial genera of the mouse gut microbiota and their relative abundances are different from those of human.<sup>14,23,24</sup> Further research on different animal models could provide more in-depth knowledge on the associations of gut microbiota and diabetes.

In this study, we established a Bama mini-pig diabetic model and performed 16S rRNA gene sequencing. The results showed that, like human and mouse, the gut microbiota of Bama mini-pig were also dominated by Firmicutes (64.24%) and Bacteroidetes (24.20%). Compared with the chow group, the DM group had a relative higher abundance of Firmicutes, represented by the genera *Lactobacillus*, *Peptococcus*, *Coprococcus\_3*, and *Clostridium\_sensu\_stricto\_1*, and a lower abundance of Bacteroidetes, represented by the genera of *Bacteroidales\_RF16\_group*, *Prevotella\_7*, *Rikenellaceae\_RC9\_gut\_group*, *p\_2534\_18B5\_gut\_group*, and *Clostridiales\_vadinBB60\_group*. As similar changes have been observed in mice on high-fat diets,<sup>25</sup> we speculated

that the reduced ratio of Bacteroidetes to Firmicutes in this mini-pig model was also mainly due to the treatment of HFD. At the genera level, we identified increases in *Lactobacillus*, *Clostridium*, and *Coprococcus\_3*, similar to those of diabetic patients.<sup>26</sup> Functionally, *Coprococcus\_3* and *Paludibacter* were reported to be correlated with host energy metabolism.<sup>27,28</sup> *Clostridium\_sensu\_stricto\_1* had a positive correlation with creatine levels, which could decrease insulin sensitivity and significantly increase the risk of type 2 diabetes mellitus (T2DM).<sup>29,30</sup> Meanwhile, *Rikenellaceae* and *Clostridiales\_vadinBB60\_group*, which were inversely correlated with blood glucose and insulin resistance, had decreased proportions in DM group. The microbial composition is affected by the host genetic background and rearing environment. This study preliminarily examined the composition of the gut microbiota of diabetic mini-pig; however, the low number of each group limited the subsequent analysis. Further research on the gut microbiota of diabetic mini-pig should be performed with greater sample sizes.



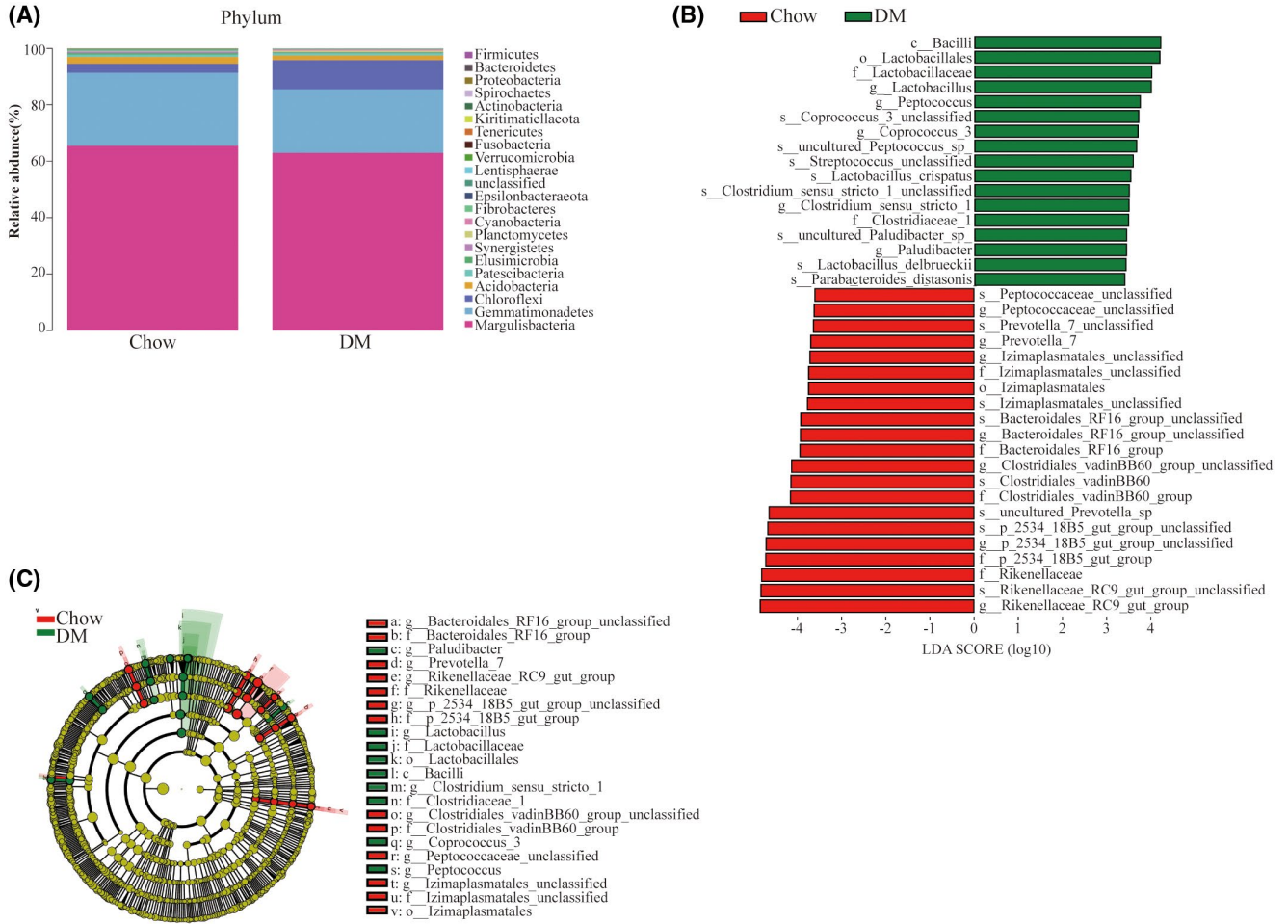


FIGURE 3 Distributions of the intestinal microbiome in the chow and DM groups. (A) Relative abundance levels of bacterial genera. (B) LfSe analysis providing phylum-to-species phylogenetic distribution. (C) LDA scores identifying differential entities between the 2 groups (LDA score  $\geq 3.0$ )

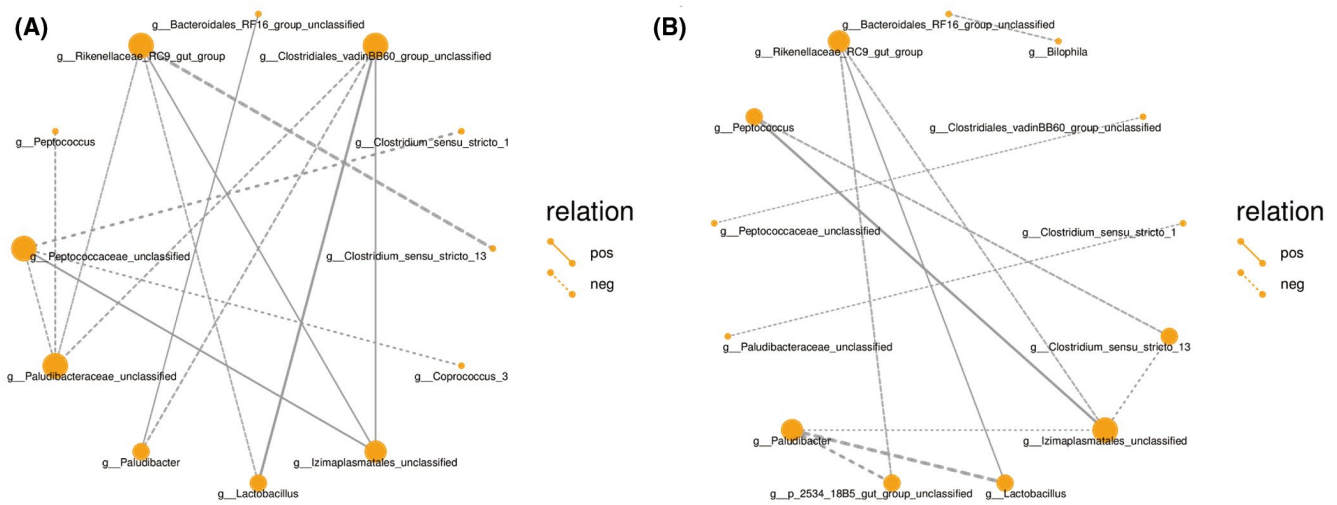


FIGURE 4 Correlation network analysis of the 30 top genera in the (A) chow group and (B) DM group. Between-node lines indicate significant Spearman correlations, with intensities reflecting their correlation coefficients (solid line, positive; dotted line, negative)

95% confidence intervals

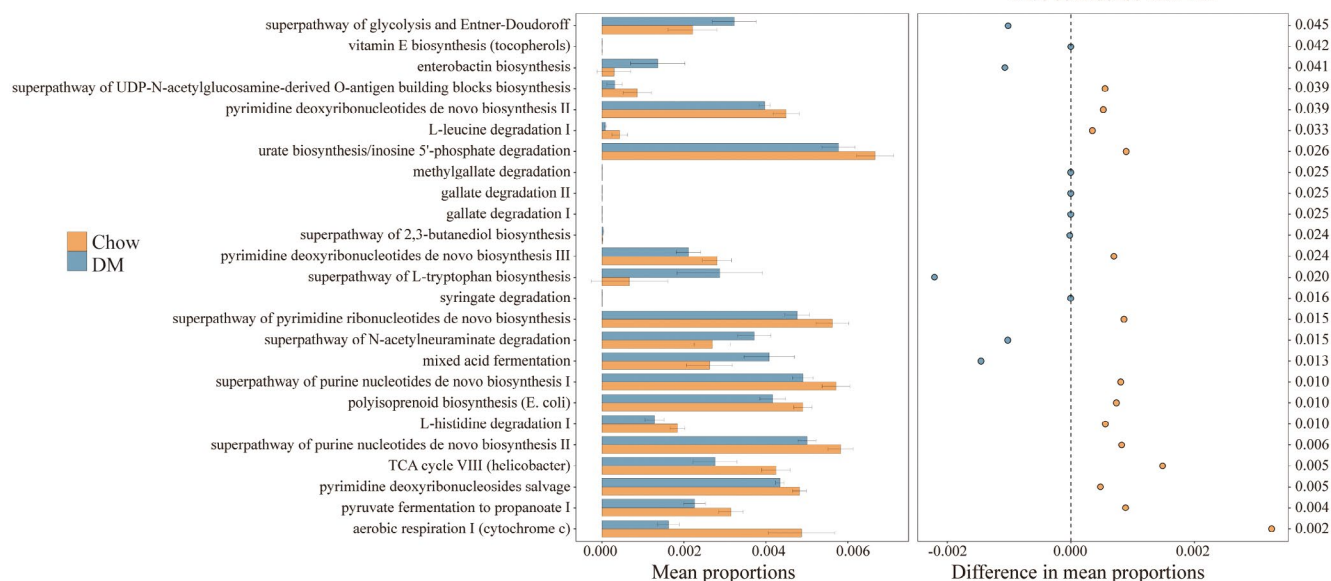


FIGURE 5 PICRUSt2 pathway analysis of the chow and DM groups

In conclusion, this study examined the gut microbiota of diabetic mini-pig and identified some changes similar to those observed in mouse and human. Further research on the gut microbiota and underlying functions are necessary and might provide novel insights into the application of mini-pigs in gut microbiota research.

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#### CONFLICT OF INTEREST

All authors read and approved the final manuscript. The authors declare no conflict of interest, and there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

#### AUTHOR CONTRIBUTIONS

Hua Chen and Miaomiao Niu designed and directed the project and analyzed data. Miaomiao Niu, Yuqiong Zhao, Lei Xiang, Yunxiao Jia, Xin Dai, and Hua Chen performed the experiments. Chen Hua is the guarantor of this work, and takes responsibility for the integrity of the data and the accuracy of the data analysis.

#### ORCID

Hua Chen  <https://orcid.org/0000-0003-2779-275X>

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