Propolis modulates the gut microbiota and improves the intestinal mucosal barrier function in diabetic rats

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ABSTRACT

Objective: Diabetes mellitus is associated with gut microbiota disturbance and intestinal mucosal injuries. This study investigated the influence of propolis on the gut microbiota and intestinal mucosa in rats with diabetes.

Methods: Sprague-Dawley (SD) rats were randomly assigned to the control group, model group, and three propolis groups (supplemented with 80, 160, and 240 mg/kg·bw propolis, respectively). A high-fat diet combined with a streptozotocin (STZ) abdominal injection were used to induce diabetes in the rats. After 4 weeks, the intestinal histopathological analysis of the ileum was observed by transmission electron microscopy. The fasting blood glucose (FBG), plasma insulin, glucose tolerance (OGTT) and glycosylated hemoglobin (HbA1c) levels were measured. The expression of tight junction (TJ) proteins in the ileum was measured using western blotting. The molecular ecology of the fecal gut microbiota was analyzed by 16S rDNA high-throughput sequencing. The contents of the short-chain fatty acids (SCFAs) in feces were measured using high-performance liquid chromatography (HPLC).

Results: After propolis treatment, compared to the model group, FBG and HbA1c levels declined, while the glucose tolerance and insulin sensitivity index (ISI) increased. The levels of TJ proteins in the ileum increased in the propolis groups. The tight junctions and gap junctions of the intestinal epithelium were also improved in the propolis groups. The contents of the feces acetic acid, propionic acid and butyrate were increased in the propolis groups. 16S rDNA high-throughput sequencing revealed that the composition of the gut microbiota of rats in the propolis supplement group was significantly improved.

Conclusions: Compared to the model group, propolis exerted hypoglycemic effects in diabetic rats, and it repaired intestinal mucosal damage, benefited the communities of the gut microbiota and increased SCFA levels in diabetic rats.

1. Introduction

Diabetes mellitus (DM) is a chronic disease and has long been defined as a heterogeneous group of metabolic disorders characterized by fasting hyperglycemia and glucose intolerance. Many health problems, including DM, can be influenced by the gut microbiota. Previous studies suggest the importance of the gut microbiota as an environmental factor, and the gut microbiota have been thought to play an important role in the development of DM [1–3].

Recent studies analyzing the gut metagenome have indicated that the dysbiosis of the human gut microbiota is associated with DM [4,5]. Sedighi M, et al [6] compared the composition of the intestinal microbiota in adult patients with type II diabetes and healthy individuals. The authors found that there were significant alterations in the dominant fecal bacterial genera in diabetic patients compared to those in the controls. Fecal microbiota transplants from healthy individuals to individuals with metabolic syndrome via a duodenal cannula improved insulin sensitivity, in association with changes to the fecal microbiota [7]. Moreover, gut microbiota can regulate the fermentation and absorption of dietary polysaccharides and may play a crucial role in small intestinal mucosal permeability. Other rodent studies, however, suggest that increased gut permeability may be a cause rather than a
consequence of diabetes mellitus [8,9]. These findings are valuable for developing approaches to control diabetes mellitus by modifying the gut microbiota.

Propolis contains a variety of components, such as flavonoids, terpenes, phenolic acids, β-steroids, and the derivatives of sesquiterpenes, naphthalene and stilbenes. Propolis is well known to perform many functions and has antioxidant, antiviral, antimicrobial, anti-inflammatory, antiatherogenic, and anticancer activities. Previously, propolis was demonstrated to potentially improve glucose metabolism and antioxidant function in diabetic rats and DM patients [10–12]. Treatment. A recent study revealed a significant increase in gut microbiota diversity after 21 days of 0.3% propolis supplementation in rats fed a Western diet [13]. Propolis modulates gut microbiota, reduces endotoxemia and TLR4 pathway expression in mice fed a high-fat diet [14].

However, whether propolis can alter the gut microbiome and the intestinal mucosa in DM rats is still unclear. Thus, this study investigated the influence of propolis on the gut microbiota and intestinal mucosa in rats with diabetes. In this study, a high-fat diet combined with streptozotocin (STZ) abdominal injection was used to establish a diabetic rat model, and diabetic rats were supplemented with different doses of propolis. The effects of propolis on blood glucose, the basic structure and function of the intestinal mucosa, the molecular ecology and the metabolites, such as short-chain fatty acids (SCFAs), of the gut microbiota were investigated.

2. Materials and methods

2.1. Collection and extraction of propolis

The Propolis used in this study was obtained from the Bee Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China. It was an ethanolic extract of raw propolis and prepared as described by Zhang H, et al [15]. The raw propolis was collected from Shandong, China, and the botanical origin was poplar (Populus sp.). The propolis extract was diluted to the required dilutions (weight to volume) using 10% ethanol. The extracts were stored at 2–8 °C in the dark and was warmed to room temperature just before injection.

Total flavonoids content was determined by high-performance liquid chromatography (HPLC). 0.2843 g of propolis extract sample was weighed and placed in a 50 mL polypropylene centrifugal tube. 25 mL methyl was added, and the sample was thoroughly shaken and swirled on the vortex mixer for 20 s. The volume was fixed to the scale with 60% methanol + water. After fully whirled, the sample solution was determined by a high-performance liquid chromatographer (Agilent 1260, Santa Clara, CA, USA). Instrument parameters: chromatographic column, C18; column length, 150 mm; inner diameter, 4.6 mm; particle size, 5 μm; mobile phase, methanol + water (58 + 42 in volume); detection wavelength, 270 nm and injection volume, 10 μL.

HPLC analysis has shown that the propolis extract contained 3.27 g of total flavonoids/100 g, including rutin 0.081 g, myricetin 0.077 g, quercetin 0.176 g, camphenanthrol 0.044 g, apigenin 0.076 g, pinus genistein 0.452 g, caustin1.739 g and galangin 0.625 g (S1 Fig.).

2.2. Animals and treatments

The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA). Animal experimental procedures were approved by the Review Committee for the Use of Human or Animal Subjects of Qingdao University of Medicine, and all efforts were made to minimize suffering.

Sixty male Sprague-Dawley (SD) rats (six-weeks-old, 160–180 g) were purchased from Shandong Lukang Laboratory Animal Center (Qingdao, China).

Rats were maintained in our animal facility at a set temperature (22–25 °C) and humidity (50%-60%) and were on a 12-h light-dark cycle for one week. Rats were caged individually and randomly allocated to the cages. After 12 h of food deprivation, the fasting blood glucose (FBG) in blood drawn from the tail was measured to determine the basal blood glucose. The animals were randomly divided into five treatment groups [n = 12/group] based on their blood glucose values, including the control group, model group and three propolis treatment groups.

The rats in the model group and three propolis treatment groups were fed a high-fat diet (lard 10%, sucrose 20%, egg yolk powder 15%, cholesterol 1.2%, pig bile salt 0.2%, and maintenance fodder 53.6%, Shandong Lukang Laboratory Animal Center.

The rats in the control group were fed a standard diet (Shandong Lukang Laboratory Animal Center, Qingdao, China). After 3 weeks, diabetes was induced by a single intraperitoneal (ip) injection of 1% streptozotocin (STZ, Solarbio Technology Co. LTD, Beijing, China) at a dose of 40 mg/kg·bw (body weight). STZ was dissolved in citric acid buffer (0.1 mmol/L, pH 4.4). The rats in the control group were intraperitoneally injected with the same dose of citric acid buffer alone.

Animals were identified as diabetic on the basis of their FBG levels (higher than 11.1 mmol/L) in blood drawn from the tail one week after STZ treatment.

Then, the diabetic rats in the propolis treatment groups were orally supplemented with 80, 160, or 240 mg/kg·bw/day propolis by gavage. The rats in the control and model groups were orally supplemented with soy bean oil at the same dose. The rats continued to receive their original diet throughout the whole experimental period. After 4 weeks of treatment, fresh feces were collected and immediately stored at −80 °C for subsequent analysis. After 12 h of food deprivation, the FBG in the blood drawn from the tail of the rats was measured. Then, the rats were orally supplemented with 2.0 g/kg·bw glucose by gavage, and the blood glucose in blood drawn from the tail was measured after 0.5 h and 2 h. Finally, the rats were anesthetized with 3% sodium pentobarbital solution, and then blood samples were collected by abdominal aorta puncture. Blood plasma and red blood cells were harvested and stored at −80 °C and 4 °C, respectively. The ileum tissues were excised.

2.3. Body weight, water intake, food intake, blood glucose and whole blood HbA1c

Body weight was recorded each week. Water intake and food intake were monitored on a daily basis during the experimental period. Fasting blood glucose levels were determined using an Accu-Chek Performa Blood Glucose Monitor Diabetes Meter and blood glucose test strips (Shanghai Roche Testing Products Co. LTD, Shanghai, China). Whole blood hemoglobin A1c (HbA1c) was measured by a high-performance liquid chromatography (HPLC) method (Variant II, Bio-Rad Laboratories, Hercules, CA, USA). The area under the blood glucose concentration curve (AUC) was calculated.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The levels of plasma insulin were assessed by ELISA using commercial kits (Cloud-Clone Corp, Houston, USA) according to the manufacturer's instructions. The insulin sensitivity index (ISI = 1/ FBG × insulin) was calculated, and the values were transformed by taking the natural logarithm (ln).

2.5. Pathological observation of the intestinal tissue

The histological change of ileum tissues was observed by Hematoxylin and eosin (HE) staining. After the animals were sacrificed, a small portion of ileum tissues was fixed in 10% buffered formalin for 24 h and embedded with paraffin. After dehydrated with serial alcohol (70%, 90%, Absolute), the samples were sectioned to 5 μm thickness and underwent hematoxylin and eosin staining. Observation was
ileum tissues were quickly excised, cut into 1 mm × 2 mm sections, to study the structural changes of ileum tissues. After the animals were sacrificed, the ileum sections were fixed with 2.5% glutaraldehyde at 4 °C for 2 h. After washing with PBS (pH 7.4) three times, the tissues were fixed with 1% osmic acid at 4 °C for 80 min and then dehydrated with a gradient series (30%–100%) of acetone. The tissues were embedded with ethoxyline resin. Then, the ultrathin sections (50–70 nm) were cut with an Ultracut E ultramicrotome (Reichert-Jung, Austria) and stained with 3% uranyl acetate and lead citrate for 30 min and 15 min, respectively. Finally, the ultrastructure of each section was examined using a JEM-1200 transmission electron microscope (JEOL, Tokyo, Japan).

2.6. Western blot analysis

The ileum tissues were excised and homogenized with a tissue protein extraction reagent (T-PER, Pierce Biotechnology, USA) according to the manufacturer’s protocol. The quantity of total protein was measured with a Bio-Rad protein assay kit (Bio-Rad Laboratory, California, USA). The protein extracts were subjected to separation on 10% SDS-polyacrylamide gels and electro-transferred to polyvinylidene fluoride (PVDF) membranes (Solarbio Science & Technology, Beijing, China).

After blocking with 5% nonfat milk in Tris buffered saline with Tween-20 detergent (TBST) for 1 h, the membranes were incubated with specific primary antibodies overnight at 4 °C. Following three washes with Tris-buffered saline (TBS), the membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. Membranes were washed 3 times with TBS, and the detection was carried out with a chemiluminescence detection kit (Santa Cruz Biotechnology, Dallas, USA) according to the manufacturer’s instructions. Quantification of the protein band intensity was performed using a UVP GDS-8000 gel imaging system (UVP, California, USA), and the results are expressed as the ratio of the relative intensity of the target proteins to that of the internal standard.

Anti-Claudin, anti-Occludin and anti-Zonula occludens protein 1 (ZO-1) were purchased from Cell Signaling Technology (Beverly, MA, USA), and the corresponding secondary antibodies were purchased from Zymed Laboratories (South San Francisco, California, USA). Anti-β-actin and a corresponding secondary antibody were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2.7. Measurement of Short-chain fatty acids (SCFAs)

SCFA concentrations in fecal samples were measured by HPLC [16]. A total of 300 mg of feces for each sample was diluted with distilled water at a 1:9 dilution. The sample was vortexed for 1 min and centrifuged at 12,000 r/min for 5 min. The supernatant was removed and filtered with a 0.2 μm thin film filter. The automatic sampler took 20 μL of sample and injected it into a high-performance liquid chromatograph (Agilent 1260, equipped with a diode-array detector and chemstation workstation, ZORBAX SB-C18 column, 4.6 mm × 250 mm × 5 mm). Mobile phase: after isocratic elution for 10 min with 0.1% phosphoric acid solution:methanol = 97.5: 2.5 (V/V) ratio, the methanol phase was increased to 100% with a short time gradient and was equilibrated for 5 min. Then, the mobile phase was adjusted with 0.1% phosphoric acid solution:methanol = 97.5: 2.5 (V/V) and was equilibrated for 5 min. The column oven temperature was 40 °C. The detection wavelength was 210 nm. To construct the SCFA calibration curves, acetic acid, propionic acid, isobutyric acid, butyric acid, isopentanoic acid and pentanoic acid standard solutions were prepared at 0.5–3.0 mg/L (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L).

2.8. Fecal DNA extraction and 16S rDNA gene sequencing

The 16S rDNA gene high-throughput sequencing procedure was performed by the Reallbio Genomics Institute (Shanghai, China). The fecal microbiome for 17 fecal samples collected from six rats in the control group, five rats in the model group and six rats in the 240 mg/kg bw/day propolis treatment group were examined using an Illumina HiSeq 2500 platform.

Total genomic DNA was extracted from frozen feces using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The microbial 16S rDNA was amplified using a 341 F/806R primer set (341 F: ACTCCTACGGGAGGCAGCAG; 806R: GGACTACHVGGGTWTCTAAT) targeting the V3-V4 region. The amplified PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, US) were pooled with an equal concentration and sequenced on an Illumina MiSeq platform (Illumina, San Diego, US) to generated paired-end reads of 300 bp. The raw data were then subjected to a quality control procedure using UPARSE [17]. The high-quality reads were then annotated to operational taxonomic units (OTUs) at the 97% similarity level using Usearch in the QIME software [18].

A representative sequence of each OTU was further assigned to the taxonomy-based analysis in the RDP (Ribosomal Database Project) database by the RDP classifier version 2.2 [19]. The relative abundance of each OTU was determined at the phylum, class, order, family and genus levels. Principal components analysis was performed using R 3.1.0 [20]. Alpha diversity (Chao1 and Shannon) and beta-diversity (principal coordinate analysis, PCoA) were analyzed using QIME software. In addition, linear discriminant analysis (LDA) effect size (LEfSe) analyses were performed with the LEfSe tool to calculate the OTU abundance and determine the differences among groups [21].

2.9. Statistical analysis

Variance analyses were performed with ANOVA with Tukey’s post hoc test. Principal component analysis and heat map analysis were conducted with R 3.1.0. Spearman’s correlation coefficients were calculated using PASW Statistics 19.0. Redundancy analysis was performed using CANOCO 4.5. Differences with P values < 0.05 were considered significant.

3. Results

3.1. Animal characteristics

The rats in the control group gradually gained weight. The food intake, water intake and behavior of these animals showed no abnormality, and their fur was smooth and glossy. The rats in the model group were lethargic, and their fur was dull and disorderly.

At the beginning of the intervention, the body weight of the rats was higher in the model group than the control group, but there was no statistical significance (P > 0.05). Then, the body weight in the model group decreased gradually and was significantly lower than that of the control group at the end of the intervention (P < 0.05). At the same time, the water intake of the model group was higher than that of the control group. After propolis treatment, the body weight of the rats increased, and there was a significant difference between the 240 mg/kg bw propolis group and the model group (P < 0.05). The water intake in the 160 and 240 mg/kg bw propolis groups was significantly lower than that of the model group (P < 0.05). In the fifth week of the experiment, the food intake in the model group was higher than that in the control group (P < 0.05), while in the propolis treatment group.
groups, the food intake was decreased compared with that in the model group \((P < 0.05)\). However, the food intake in the model group and the 80 mg/kg·bw propolis group decreased gradually. Finally, the food intake in the model group and propolis treatment groups was lower than that in the control group \((P < 0.05)\). The food intake in propolis treatment groups was slightly higher than that in the model group, but there was no statistical significance \((P > 0.05)\). Fig. 1 shows the body weight, water intake and food intake of the rats in the different groups.

3.2. Effects of propolis on FBG, glucose tolerance and insulin levels in diabetic rats

As shown in Fig. 2, the fasting blood glucose (FBG), AUC and HbA1c levels were significantly increased in the model group, while the insulin sensitivity index (ISI) was decreased, compared with those in the control group. Compared to the model group, propolis treatment reduced the FBG, AUC and HbA1c levels and increased the glucose tolerance (OGTT) and ISI \((P < 0.05)\), but there were no differences among the three propolis treatment groups. In OGTT, the blood glucose levels of the model and propolis treatment groups in 0 h, 0.5 h and 2 h were higher than those of the control group \((P < 0.05)\), but propolis treatment especially in the 160 mg/kg·bw and 240 mg/kg·bw group decreased the blood glucose levels in 0.5 h and 2 h of OGTT compared to the model group \((P < 0.05)\). There was no significant difference in plasma insulin levels among all groups \((P > 0.05)\). Because the ISI was inversely proportional to insulin resistance, the data showed that insulin resistance was occurred in rats of the model group, but reduced after propolis treatment.

3.3. Pathological changes of the ileum tissue based on electron microscopy

HE staining showed the pathological changes of ileum tissues and indicated that the ileum structure was normal, and intestinal villi were lined in neat rows in the control group. However, in the model group
The villi were irregular with local epithelial shedding. Propolis treatment improved the mucosal structure of ileum tissues, and the intestinal villi were arranged neatly, with only a small amount inflammatory cell infiltration observed (Fig. 3A). The length of the intestinal villi in the model and 80 mg/kg·bw propolis group were both shorter than that in the control group. 240 mg/kg·bw propolis treatment increased the length of the intestinal villi (Fig. 3B).

The intercellular junction mainly includes tight junctions, adherens junctions, desmosomes, gap junctions, etc. The tight junctions can close the intercellular space and prevent solute flux across both the endothelial and epithelial barriers [22]. Adherens junctions and desmosomes connect the cytoskeletal elements of two neighboring cells through transmembrane cadherins. Gap junctions provide a platform for small molecule exchange between neighboring cells.

In transmission electron microscopy, the intercellular junctions between cells were complete and clearly visible in the control group. In the model group, the tight junctions showed slight relaxation, and the electron density was reduced compared to that in the control group. Meanwhile, widened block gaps were observed in the adherens junctions and gap junctions of the intestinal epithelium of the model group compared to the control group. After the propolis intervention, the tight junctions and gap junctions of the intestinal epithelium were improved, and the narrowing of the intercellular spaces was observed compared to those in the model group (Fig. 3C). These results suggested that abnormal morphological and pathological changes happened in the intestinal mucosal epithelial cells of diabetic rats and that propolis treatment could repair the intestinal mucosal injury.

Fig. 2. Effects of propolis on the FBG, glucose tolerance and insulin levels in diabetic rats. A) Fasting blood glucose; B) HbA1c; C) Serum insulin; D) ISI; E) Glucose tolerance; F) Blood glucose analysis at different time points of glucose tolerance test; G) Area under the curve of blood glucose (AUC). The fasting blood glucose (FBG), AUC and HbA1c levels were significantly increased in the model group, while the insulin sensitivity index (ISI) was decreased, compared with that in the control group. Propolis treatment especially in the 160 mg/kg·bw and 240 mg/kg·bw group reduced the FBG, AUC and HbA1c levels and increased glucose tolerance (OGTT) and ISI, but there were no differences among the three propolis treatment groups. There was no significant difference in plasma insulin levels in all groups. Data are represented as the mean ± SD. n = 12 in each group. Note: *P < 0.05 vs Control; △P < 0.05 vs Model.
3.4. Effects of propolis on TJ protein levels

Occludin, claudins and junctional adhesion molecules are important components of tight junctions in intestinal barrier function. These transmembrane proteins are linked to the cytoskeleton by zonula occludens to constitute a tight junction.

To further evaluate the intestinal mucosal barrier function, the expression of TJ proteins in the ileum tissue of diabetic rats was determined by western blotting. As shown in Fig. 3D, in the model group, the expression levels of Claudin, Occludin, and ZO-1 protein were decreased compared with those in the control group. After propolis treatment, the levels of TJ proteins were increased, and there was a dose-dependent effect ($P < 0.05$).

3.5. The contents of SCFAs in the feces

As shown in Fig. 4, the levels of acetic acid, propionic acid and butyric acid in the feces of the model group were significantly lower than those of the control group ($P < 0.05$), while there was no significant difference in the contents of pentanoic acid, isobutyric acid and isovaleric acid between the two groups ($P > 0.05$). After propolis treatment, the contents of acetic acid, propionic acid and butyric acid were significantly increased compared with those of the model group ($P < 0.05$).

3.6. Effect of propolis on the gut microbiota microecology in diabetic rats

Based on the GTTs and all the other above data, 240 mg/kg propolis treatment had most significant effect on lowering blood glucose and improving intestinal mucosal barrier function. So high-throughput sequencing of fecal samples from the control, model and 240 mg/kg·bw Propolis treatment groups was performed. The 16S rDNA high-throughput sequencing (V3-V4 region) was performed using an Illumina MiSeq platform. After assembly, quality filtering and the random extraction of sequences at 97% similarity, the OTUs for species classification were obtained.

3.6.1. Alpha and Beta diversity analysis

The alpha diversity analysis of the species distribution is shown in Fig. 5A. Alpha diversity analysis evaluates the species diversity of a single sample, including the Chao1, observed species, Shannon and Simpson indexes. The Chao1 and observed species are used to estimate
the species richness; the Shannon index and Simpson index are diversity indexes. When the Shannon index is larger and the Simpson index is closer to 0, it means that there are abundant species in the sample. Our results showed that the Chao1 value and observed species value were significantly different among the three groups, which suggested that there were significant differences in gut microbiota abundance. However, there were no significant differences in the Shannon value and Simpson value among the groups. Therefore, the diversity of the samples in each group was not affected.

Beta diversity analysis was then performed based on a weighted UniFrac analysis. The OTU abundance matrix obtained from the three groups was subjected to principal coordinate analysis (PCoA) to evaluate the similarities among the samples and groups. The PCoA scores showed a clear separation of the model group from the control group and the propolis treatment group (Fig. 5B).

To evaluate the statistical analysis of the differences among samples, a cluster analysis was carried out, and the distance between the samples was calculated to determine the similarity of the species composition of the samples. The closer that the samples are in this analysis, the more similar the species compositions between the samples are. The beta diversity heatmap (weighted) shows that the diabetic rats with propolis treatment had similar bacterial compositions to those in the control group (Fig. 5C). ANOSIM analysis of variance confirmed that there was a statistically significant separation of the three groups ($R = 0.465$, $P < 0.05$, Fig. 5D). Propolis treatment shifted the overall structure of the gut microbiota in diabetic rats toward that in normal rats.

### 3.6.2. The microbial community structures at the phylum, class, order, family and genera levels

According to the results of the species annotations, there were significant differences in the relative abundances of species at the phylum, class, order, family and genera levels from the different groups, and the relative abundance of species at the phylum, family and genera level of the three groups are shown in Fig. 6, S2 Excel and S3 Excel.

Firmicutes, Actinobacteria, and Bacteroidetes comprised the main phyla in each group, but the composition was different (Fig. 6A). The abundances of Firmicutes in the model and propolis groups (78.92% and 70.92%, respectively) were lower than that in the control group (85.36%), but there was no statistically significant difference among the groups ($P > 0.05$), while the abundances of Actinobacteria (18.31% and 27.42%, respectively) were increased compared with those in the control group (7.05%, $P < 0.05$). The abundances of Bacteroidetes in the model and propolis groups (1.62% and 0.77%, respectively) were lower than those in the control group (6.02%, $P < 0.05$).

At the family level, the predominant bacterial family was Lachnospiraceae from the Firmicutes phylum in the control and propolis groups (each 48.69%), while the abundances of Lachnospiraceae and Lactobacillaceae declined, with abundances of 19.58% and 6.69%, respectively. Enterococcaceae from the Firmicutes phylum were enriched in the model group (12.03%), while the abundances of Enterococcaceae in the

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**Fig. 4.** The contents of the SCFAs in the feces. A) Standard short-chain fatty acid liquid chromatography (3.0 mg/L). The correlation coefficient of the calibration curve is $> 0.99$. B) The contents of the short-chain fatty acids. The levels of acetic acid, propionic acid and butyric acid in the feces of the model group were significantly lower than those of the control group. After propolis treatment, the levels of acetic acid, propionic acid and butyric acid were significantly increased compared with those of the model group. 1: acetic acid; 2: propionic acid; 3: isobutyric acid; 4: butyric acid; 5: isovaleric acid; 6: pentanoic acid. *$P < 0.05$ vs Control group; △$P < 0.05$ vs Model group.
The propolis group and control group declined to 4.94% and 0.19%, respectively \( (P < 0.05, \text{Fig. 6B}) \). We also calculated the abundance ratio of Bacteroidetes/Firmicutes. In the control group, this ratio was 0.07, which was higher than that in the model group and propolis group \( (0.02 \text{ and } 0.01, \text{respectively} \; (P < 0.05)) \).

We found that there were 36 species of bacterial genera, and their abundances were significantly different among the three groups. There were significant alterations in the distribution of the intestinal bacterial genus among the three groups. The predominant bacterial genus in the control and propolis groups was Lactobacillus, with an abundance of 32.51% and 19.24%, respectively, but the abundance of Lactobacillus in the model group declined to 8.42\( \% \) \( (P < 0.05) \). At the same time, there was a significant downregulation of the abundances of Blautia, Fusicatenibacter and Clostridium X1Va in the model group compared with the control and propolis groups \( (P < 0.05) \). These species are all from the Firmicutes phylum. Among them, Lactobacillus is from the Lactobacillaceae family, and the remaining three species are from the Lachnospiraceae family. The predominant bacterial genus in the model group was Streptococcus, with an abundance of 33.50\( \% \), which declined to 3.33\( \% \) and 13.54\( \% \) in the control and propolis groups, respectively \( (P < 0.05) \). Meanwhile, Enterococcus was enriched in the model group (12.68\%), while the abundances of Enterococcus in the propolis group and control group declined to 5.59\% and 0.24\% respectively \( (P < 0.05) \). The abundances of Clostridium sensu stricto, Turicibacter and Arthrobacter were significantly higher in the model group than in the control group. After propolis treatment, the abundances of the abovementioned species were lower than those in the model group \( (P < 0.05) \). In the cluster analysis of the top 20 abundance bacterial genera, it was found that the distribution of these genera in the propolis group was closer to that in the normal control group than in the model group (Fig. 6C). Linear discriminant analysis (LDA) coupled effect size measurements (LEfSe) were used to analyze whether the gut microbiota differed significantly among groups from the phylum level to the genera level (Fig. 6D). These findings indicated that the microbial community structure in diabetic rats differed from that in normal rats. Propolis treatment could modify the dysbacteriosis caused by diabetes and restore the homeostasis of the gut microbiota microecology.
Correlation between the abundances of differentially bacterial genera and biochemical parameters

To explore the main drivers responsible for the changes in diabetic rats, the relationships between FBG, HbA1c, SCFAs and the abundances of differentially bacterial genera were assessed (Table 1). The results showed that the abundances of Streptococcus, Enterococcus, Clostridium sensu stricto and Turicibacter were positively correlated with...
Table 1: Correlation between differentially bacteria genus and biochemical parameters.

<table>
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<th>Genera</th>
<th>FBG p</th>
<th>FBG r</th>
<th>HbA1c p</th>
<th>HbA1c r</th>
<th>Claudin p</th>
<th>Claudin r</th>
<th>Occludin p</th>
<th>Occludin r</th>
<th>ZO-1 p</th>
<th>ZO-1 r</th>
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<th>Acetic acid r</th>
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<td>−0.51</td>
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<td>0.40</td>
<td>0.11</td>
<td>0.34</td>
<td>0.18</td>
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</table>

Significant correlations (P < 0.05) are in bold.

FBG and HbA1c levels. The abundances of Lactobacillus, Blautia, Fusobacterium, Clostridium XIVa, Porondaea and Clostridium IV were negatively correlated with FBG and HbA1c levels (P < 0.05). At the same time, the abundances of Lactobacillus, Blautia and Clostridium XIVa were significantly positively correlated with the content of the SCFAs, such as acetic acid, propionic acid and butyric acid, while the abundances of Streptococcus, Enterococcus and other bacteria were significantly negatively correlated with the content of the SCFAs (P < 0.05).

The levels of Claudin, Occludin, and ZO-1 were negatively correlated with the abundance of Streptococcus, and positively correlated with the abundance of Streptococcus, and negatively correlated with the abundance of Streptococcus, and positively correlated with the abundance of Streptococcus (P < 0.05).

4. Discussion

Propolis, a natural resinous substance made by bees from material extracted from plants, flowers and bee wax, has shown great therapeutic effects and has been widely used in the food and drug industries. In this study, a high-fat diet combined with STZ abdominal injection induced diabetes in rats. Propolis treatment reduced the FBG and HbA1c levels and increased the OGTT and ISI in diabetic rats. Our data showed that propolis had the ability to prevent hyperglycemia, which was consistent with previous studies [23–25].

The composition of the gut microbiome and the richness of certain bacteria are important factors modulating diabetes development [5,26]. In this study, the analysis of the gut microbiota molecular ecology by 16S rDNA high-throughput sequencing technology showed that there were gut microbiota disorders in diabetic rats. There were significant differences in the gut microbiota abundance between diabetic rats and normal rats. The OTU abundance decreased significantly, and the composition of the gut microbiota changed in diabetic rats compared to those in the controls. After similarity and cluster analysis, we found that the flora composition in the propolis intervention group was closer to that in the control group, and the hypoglycemic effect of propolis was related to the improvement of the disorder of the gut microbiota in diabetic rats. This may be one of the mechanisms of propolis-induced hypoglycemia. Many current studies to assess the potential of probiotics in honey suggest that honey may improve the gut microbial balance in addition to the bactericidal activity [27]. Wang K, et al [28] found that Brazilian propolis increased the diversity and richness of gut microbiota populations against dextran sulfate sodium (DSS)-induced colitis in rats.

The gut microbiota is mainly composed of the phyla Actinobacteria and Proteobacteria and approximately 90% are from the Bacteroidetes and Firmicutes phyla [29]. Our data showed that Firmicutes, Actinobacteria, and Bacteroidetes comprised the main phyla in diabetic and nondiabetic rats. Rats with diabetes showed a significant shift in the composition of gut microbiota, with a reduced percentage of Firmicutes.

Table 2: Correlation between differentially biochemical parameters.

<table>
<thead>
<tr>
<th>Index</th>
<th>Claudin p</th>
<th>Claudin r</th>
<th>Occludin p</th>
<th>Occludin r</th>
<th>ZO-1 p</th>
<th>Acetic acid p</th>
<th>Acetic acid r</th>
<th>Propionic acid p</th>
<th>Propionic acid r</th>
<th>Butyric acid p</th>
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<tr>
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<td>−0.78</td>
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<td>HbA1c</td>
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<td>0.01</td>
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<td>−0.66</td>
<td>0.03</td>
<td>−0.63</td>
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</table>

Significant correlations (P < 0.05) are in bold.
and Bacteroidetes and an increased proportion of Actinobacteria compared to those in the controls. There is a lack of uniformity in the proportions of the Firmicutes phylum in diabetic patients or animals. In humans, Larsen, et al [30] studied the fecal bacterial composition of 36 adult males. The proportions of the Firmicutes phylum were significantly reduced in the diabetic group compared to those in the healthy controls. Lau K, et al [31] reported that the proportions of Firmicutes were significantly reduced in diabetic bio-breeding rats compared to those in the controls. However, diabetic leptin-resistant mice were enriched in the model group and they are closely related to vitamin C metabolism, thioctic acid metabolism, glycolysis and glycogenogenesis, ion channel and phosphate transfer enzyme system metabolism. After propolis treatment, the abundances of the above species were different from model group \((P < 0.05)\), and similar to those in the control group. Blue circle: positive correlation; red circle: negative correlation. The red box in the circle indicated significant correlation between different species and metabolic pathways \((P < 0.05)\) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Furthermore, there were significant alterations in the distribution of the intestinal bacterial species at the genus level among the control, model and propolis treatment groups. Our data showed that there were 36 species of differentially abundant bacterial genera among the groups. Propolis treatment could modify the dysbacteriosis caused by diabetes and restore the homeostasis of the gut microbiota microbiology.

It is worth noting that Lactobacillus was the predominant bacterial genus in the control and propolis treatment groups, while the predominant bacterial genera in the model group were Streptococcus. After propolis treatment, the abundances of the above species were different from model group \((P < 0.05)\), and similar to those in the control group. Blue circle: positive correlation; red circle: negative correlation. The red box in the circle indicated significant correlation between different species and metabolic pathways \((P < 0.05)\) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
indicated that the distribution, rather than the abundance, of the bacterial genera in the Firmicutes phylum was closely linked to the pathogenesis of diabetes.

Moreover, we found that after propolis treatment, the contents of the SCFAs were significantly altered. The abundances of Lactobacillus, Blautia and Clostridium X1Va were significantly correlated with the content of acetic acid, propionic acid and butyric acid, while the abundances of Streptococcus, Enterococcus and other bacteria were significantly negatively correlated with the content of the SCFAs.

The beneficial effects of the probiotic Lactobacillus on the gut microbiota and glucose metabolism in healthy individuals [35] and animal diabetes models [36] were reported previously. In a randomized controlled study, probiotic Lactobacillus reduced bacterial translocation in type II diabetes mellitus [37]. Bauer PV, et al [38] found that a high-fat diet in rats decreased the abundance of Lactobacillus. Metformin treatment restored sodium glucose cotransporter-1 (SGLT1) expression and glucose sensing while shifting the upper small intestinal microbiota, partly by increasing the abundance of Lactobacillus. SCFAs are metabolic products of gut microbiota metabolism. The main SCFA products are acetate, propionate, and butyrate. Lactobacillus can produce lactic acid, which has the characteristics of probiotics. Some bacteria in the gut can turn lactic acid into butyric acid. SCFAs could also stimulate the secretion of intestine-trophic glucagon-like peptide to affect the intestines and reduce gut permeability [39,40]. Butyric acid can promote the synthesis of intestinal mucosin, which is beneficial to the repair of the intestinal mucosa and its function. Blautia and Clostridium X1Va belong to the Lachnospiraceae family, which is mainly composed of SCFA-producing genera [41]. Tong X, et al [42] found that metformin and a Chinese herbal formula may ameliorate type II diabetes with hyperlipidemia by enriching beneficial bacteria, such as Blautia.

In addition, SCFAs could improve intestinal barrier function through the reassembly of tight junctions [43]. Therefore, we examined the intestinal mucosal barrier function in diabetic rats. Based on transmission electron microscopy, the tight junctions showed slight relaxation, and the electron density was reduced in the model group compared to that in the control group. Meanwhile, widened block gaps were observed in the adherens junctions and gap junctions of the intestinal epithelium of the model group compared to the control group. These results suggested that abnormal morphological and pathological changes happened in intestinal mucosal epithelial cells. After the propolis intervention, the tight junction and gap junctions of the intestinal epithelium were improved, and the narrowing of intercellular spaces was observed. Transmembrane barrier proteins such as occludin and claudins compose tight junctions. Intracellular scaffold proteins such as ZO-1 link the transmembrane barrier proteins to actin and microtubules, which play a decisive role in the architecture as well as the physiology of the tight junctions [44]. Western blotting showed that the expression levels of Claudin, Occludin, and ZO-1 were decreased in the model group compared to the control group, while after propolis treatment, the levels of TJ proteins were increased compared to that in the model group. Our data showed that propolis treatment could repair intestinal mucosal damage in diabetic rats.

Moreover, the metabolism of diabetic patients tends to be dysfunctional and defective. Intestinal bacteria participate in the host's physiology of the tight junctions [44]. Correlation analysis between differentially bacteria genus and metabolic pathways in the current study showed that several differentially bacteria genus closely related to biotin metabolism, glycerin phospholipid metabolism and linoleic acid metabolism were enriched in the control group and propolis treatment group. Among them, Blautia and Clostridium XVIII were closely related to the degradation of propylene phenol, chlorinated paraffin, chlorine olefins and nitro toluene, cell division, cytoskeletal protein formation, and glutamatergic synapse metabolic pathway. Streptococcus and Enterococcus enriched in the model group were closely related to vitamin C metabolism, thioctic acid metabolism, glycolysis and glycogonogenesis, ion channel and phosphate transfer enzyme system metabolism. Propolis maybe improve the metabolism of diabatic rats by up-regulation Lactobacillus, Blautia and Clostridium XVIII, which had the protection and promotion function on material metabolism. But whether the above differentially bacteria genus and their related metabolic pathways was the mechanism of propolis in hypoglycemic effect was not clear and needs further study.

5. Conclusions

These findings indicated that the microbial community structure in diabetic rats differed from that in normal rats. The treatment with propolis was effective in reducing fasting plasma glucose and enhancing insulin sensitivity. Compared to the model group, propolis could improve intestinal microecology disorder, increase the metabolic products of the gut microbiota, such as short-chain fatty acids, and repair intestinal mucosal damage in diabetic rats, which may be one of the mechanisms through which propolis improves hyperglycemia.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109393.

References


