Intake of total saponins and polysaccharides from *Polygonatum kingianum* affects the gut microbiota in diabetic rats

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**Abstract**

**Background:** The gut microbiota has been reported to play a critical role in metabolic diseases, including in diabetes. *Polygonatum kingianum* has been used in the treatment of diabetes and related diseases in China for centuries. Total saponins (TSPK) and total polysaccharides (PSPK) were reported to be major types of active constituents of *P. kingianum*. This research aims at investigation of their therapeutic mechanisms on diabetes based on the regulation of gut microbiota.

**Study design:** Type 2 diabetes (T2D) rats were induced by high-fat diet (HFD) and streptozotocin-injection from male Sprague-Dawley (SD) rats. The blood biochemical indicators were measured. Intestinal microbial diversities and the overall structural changes in gut microbiota and the contents of the short chain fatty acids (SCFAs) were discussed.

**Methods:** T2D rats were treated with TSPK (0.025 and 0.1 g/kg) and PSPK (0.1 g/kg) for 56 days. Major biochemical indexes, such as fasting blood glucose (FBG), fasting insulin (FINS) and lipopolysaccharide (LPS), were measured. Intestinal microbial diversities and the overall structural changes in gut microbiota were discussed based on the sequencing results on V4 region of 16S rDNA. Moreover, the contents of the SCFAs in faeces, which were fermentation products produced from gut microbiota were determined by gas chromatography (GC).

**Results:** Oral administration of TSPK and PSPK prevented the increase of FBG. TSPK (0.025 g/kg) enhanced the content of FINS at the end of research. Furthermore, TSPK and PSPK improved the intestinal microecology by decreasing the abundances of Bacteroidetes and Proteobacteria, and increasing that of Firmicutes. However, TSPK.L, PSPK and TSPK.H displayed discrepant regulation roles on Firmicutes. TSPK.L and PSPK significantly increased the abundance of Ruminococcaceae family and Ruminococcus genus in Firmicutes phylum, however, TSPK.H increased the abundances of Veillonellaceae family and Anaerovibrio genus. 57 Key variables, altered after treated by TSPK and PSPK, correlated to the alternations of FBG, FINS, LPS and body weight were identified. In addition, TSPK.L, PSPK and TSPK.H showed different adjustment on the contents of SCFAs.

**Conclusion:** These results suggested that, compared to the normal rats, the structure of gut microbiota was significantly changed in diabetic rats. Oral administration with TSPK and PSPK could prevent T2D by its regulation role on the gut microbiota.

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**Introduction**

The gut microbiota composition and its balance have been associated with several hallmarks of metabolic syndrome, such as obesity, diabetes, cardiovascular diseases, and non-alcoholic steatohepatitis (Turnbaugh et al., 2006; Loscalzo, 2011; Mouzaki et al., 2013). There are an increasingly recognitions about the connection between gut microbiota and energy homeostasis and its role in the pathogenesis of diabetes has been elucidated these years. Growing evidences suggest that gut microbiota contributes to the onset of the low-grade inflammation, characterizing these metabolic disorders via mechanisms associated with gut barrier dysfunctions.
inflammation (Cani et al., 2007, 2008, 2009; Backhed et al., 2004). From one side, the gut microbiota helps us to digest food components and regulate host fat storage genes, thus modulating host energy homeostasis (Dandona et al., 2004; Membrez et al., 2008). From the otherside, imbalances in the structure of the gut microbiota, induced by high-fat diet (HFD) consumption, may impair gut barrier function and increase the levels of endotoxin in circulating systems, which may lead to metabolic endotoxinaemia, insulin resistance, obesity, and even diabetes (Backhed et al., 2004; Brugman et al., 2006).

The ratio of Bacteroidetes to Firmicutes phylum correlated positively and significantly with plasma glucose concentration. Furthermore, the proportions of phylum Firmicutes and class Clostridia were significantly reduced in the diabetic individuals. In the meantime, class Betaproteobacteria was highly enriched in diabetic compared to non-diabetic persons and positively correlated with plasma glucose (Larsen et al., 2010). Dietary factors, such as HFD, also induce inflammation by increasing endotoxin levels in the intestinal lumen as well as in the plasma by altering the gut microbiota composition and increasing its intestinal permeability through the induction of Toll-like receptor 4 (TLR4), thereby accelerating obesity and type 2 diabetes (Kim et al., 2012).

Medical plants have been used for years as source of traditional Chinese medicine to treat various diseases, such as diabetes. *Polygonatum kingianum* Collett & Hemsl. (Asparagaceae), is one species of Polygonati Rhizoma recorded in Pharmacopoeia, which is widely reported and used as adjuvant therapy for diabetes due to its blood sugar level lowering effects (Deng et al., 2012; Commission of Chinese Pharmacopoeia, 2015; Chen et al., 2010). Extracts of *P. kingianum* showed remarkable blood sugar levels management ability in hyperglycemic mice induced by exogenous glucose diets, adrenaline or alloxan. These effects might possibly be related to its regulation effects on serum insulin level, nitric oxide (NO) content in serum and liver tissue. Moreover, nitric oxide synthase (NOS) level was decreased significantly in *P. kingianum* treated group (Shu et al., 2009; Gong et al., 2009; Xu et al., 2009; Zhang et al., 2006).

In our previous research, TSPK showed potential α-glycosidase inhibition activities and blood glucose lowering activities (Lu et al., 2015, 2016). However, whether gut microbiota composition regulation was involved or not in its blood glucose regulation activity has not been elucidated yet. Therefore, in this research, the possible blood sugar regulation and microecological modulator activities of *P. kingianum* was carried out.

**Materials and methods**

**Collection of *P. kingianum***

The material was collected from Shalan Country, Kunming, Yunnan Province, People’s Republic of China in December 2013, and was identified as rhizome of *P. kingianum* by Associate Professor Jie Yu of Yunnan University of Traditional Chinese Medicine. A voucher specimen was deposited in the Herbarium of Pharmacognosy, Yunnan University of Traditional Chinese Medicine (Voucher specimens was DHJ-20150326).

**Preparation of total saponins from *P. kingianum* (TSPK) and determination of diosgenin in TSPK**

1000 g of powder of *P. kingianum* rhizome was refluxed twice with 80% ethanol (10×) for 1.5 h and 1 h, respectively. These extracting solutions were combined and concentrated to a final volume of 1000 ml at 60 °C, and extracted with petroleum ether, ethyl acetate and n-butanol (1000 ml for three times, respectively) to obtain the fraction of n-butanol. The fraction of n-butanol was dissolved in distilled water (0.1 g/ml) after n-butyl alcohol was removed and then purified with D101 macroporous resin. It was washed with distilled water (3×) to remove polysaccharide. Later, it was eluted with 80% ethanol (8×). The eluent was collected, condensed and lyophilized by freeze-drying (FD5–3, SIM International Group Co., Ltd, Beijing, China) and stored in -20 °C till usage. The TSPK yield was 250 mg/g crude drug.

UPLC determination of diosgenin in TSPK was carried out after acid hydrolysis. About 300 mg freeze-dried powder of TSPK was refluxed and hydrolyzed with 100 ml HCl (3 mol/l) for 8 h. Then, the hydrolysate was neutralized with NaOH (3 mol/l) to pH 7.0 and extracted with 100 ml CHCl3 for 3 times. Chloroform layer was collected, recovered and the residue was dissolved in 50 ml MeOH for the UPLC determination of diosgenin. The UPLC system consisting of an ultra-high pressure pump (LC-30A, Shimadzu), a communications bus module (CBM-20A, Shimadzu, Kyoto, Japan), a column oven (CTO-30A, Shimadzu) and a DAD detector (SPD-M20A, Shimadzu). The analytical column employed was Agilent C18 (2.1 × 250 mm ID, 1.8 μm, Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of MeOH and water (85:15, v/v), was set at a flow rate of 0.25 ml/min. The detection wavelength was set at 203 nm. The injection volume was 10 μl. The column temperature was 30 °C and the run-time was 25 min. Standard material is a commercial diosgenin (National Institutes for Food and Drug Control, Beijing, China; Production batch number: 101324963. CAS: 3767–28–0).

**Preparation and quantification of polysaccharides from *P. kingianum* (PSPK)**

1000 g powder of *P. kingianum* rhizome was refluxed twice with distilled water (15 and 10×) for 1.5 h and 1 h, respectively. These extracting solutions were combined and concentrated to a final volume of 1000 ml. The extract was diluted with 95% ethanol (4000 ml), filtered and dried under vacuum to obtain a dry mass that was extracted with ethyl alcohol, acetone, diethyl ether and then with the mixture chloroform: n-butyl alcohol (4:1). Finally, the residue was dialyzed (dialysis bag: MW(Molecular Weight): 7000, Bio Sharp Technologies, Carlisbad, CA, USA) in deionized water for 3 days and then lyophilized by freeze-drying. The PSPK yield was 40 g/kg crude drug.

According to the Chinese Pharmacopoeia (2015 edition), quantification of PSPK was conducted by anthrone-sulfuric acid colorimetric methods (Commission of Chinese Pharmacopoeia, 2015). Generally, 2 ml of distilled water and 8 ml of 0.2% anthrone-sulfuric acid solution were added to 0.5 ml PSPK (1 mg/ml). The mixture was heated in 98~100 °C for 10 min and then cooled in an ice water bath for another 10 min. Then, the absorbance at 582 nm was recorded and calculated with reference to anhydrous glucose.

**Induction of diabetic rats and treatment protocols**

Male SD rats (weighed 200 ± 20 g, aged 8 weeks) were provided by the Da Shuo Biotech Co., Ltd. Chengdu, China. They were acclimatized in the controlled environment (temperature 22 ± 1 °C; 60 ± 10% humidity; and a 12 h/12 h light/dark cycle) with free access to water and standard chow. All rats were fed for 1 week. 10 SD male rats were set as standard chow control group (CON) from the beginning. Then, all rats, except those of the normal control group, were fed with HFD (containing 1% cholesterol, 1% salt, 10% lard, 10% yolk powder, 10% cane sugar, 0.5% pig-bile salt and 67.5% standard chow) to the end of the experiment (total 86 days). All rats were intraperitoneally injected with 30 mg/kg-d streptozotocin (STZ; MP, Biomedicals, LLC, Santa Ana, CA, USA. dissolved in an ice-cold 0.1 M citrate buffer) twice, on day 16 and day 22, except the normal rats.
After induction by STZ, 50 male diabetic rats, with serum glucose concentrations higher than 11.0 mM, were randomly divided into 5 groups in the 30th day: diabetes mellitus group (DM), metformin positive control group (Met), TSPK (TSPKL and TSPKH) group and PSPK group. All rats were dosed by gavage once per day for eight consecutive weeks (from day 31 to day 86). All rats were fasted for 2 h every day before administration of therapeutic agents. A schematic diagram of the treatment schedule in this research is showed in Fig. 1.

All animal experiments were performed in compliance with the Animal Experimental Ethics Committee of Yunnan University of Traditional Chinese Medicine (Approval number: R-062014026; Approval date: July, 2014). All reasonable efforts were made to minimize the animals’ suffering.

Measurement of blood glucose and fasting insulin (FINS) levels

Blood glucose concentrations were measured from blood taken from the tail using a blood glucose meter (ACCU-Check blood glucose meter, Roche Diagnostics, Shanghai, China) at the 30th, 65th and 86th day. Blood samples of rats for other assays were obtained from angular vein in the 86th day. The blood samples were centrifuged at 6900 g for 10 min. at 4 °C. Serum was immediately collected, frozen and stored at −80 °C until analysis. Commercial enzyme-linked immunosorbent assay (ELISA) kits (WuHan HuaMei Biotech Co., Ltd., WuHan, China.) were used in FINS quantification. All analyses were performed in accordance with the manuals provided by the manufacturers.

Measurement of diversity of the colony in small intestinal tract

At the end of the treatment, rats were sacrificed using an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and then finely dissect to obtain the small intestine tissue samples. All the small intestine samples were stored at −80 °C until tested.

All the small intestine contents samples in the same group (0.5 ml for each) were carefully blended. Mixed sample (0.5 ml) was diluted in 30 ml of sterile saline and incubated at 37 °C at 180 rpm for 0.5 h using a rocking incubator (2HWY111B, Shanghai Wisdom City Analysis Instrument Manufacturing Co., Ltd., China). Then 50 μl of the supernatant was coated on blood agar plates and then incubated at 37 °C for 24 h in MJ-Mould Cultivation Cabinet (Shanghai Constant Technology Co., Ltd., Shanghai, China).

Measurement of portal vein endotoxin LPS levels

Hepatic portal vein blood samples were centrifuged at 1530 g for 10 min at 4 °C after collected under sodium pentobarbital (50 mg/kg) anesthetic condition. LPS concentrations were measured using a tachypleus amebocyte lysate test purchased from Chinese Horseshoe Crab Reagent Manufactory, Co., Ltd., Xiamen, China.

Overall structural changes in gut microbiota

At the end of the experiment, faeces of all rats were collected in sterilized plastic tubes and stored in a −80 °C until tested. All the fecal samples in the same group (0.5 mg for each) were carefully blended, and 0.5 mg of mixed samples were conducted by SDS method for genomic DNA extraction. The DNA extracted from fecal samples were chosen for pyrosequencing of V4 regions of 16S rDNA.

PCR amplification was conducted with the S15F-806R primer set, and the V4 region of 16S rDNA gene was amplified. Sequencing libraries were generated using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) following manufacturer’s recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific Waltham, Massachusetts, USA) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on IlluminaHiSeq 2500 platform and 250 bp paired-end reads were generated.

Assay of short-chain fatty acids (SCFAs) in fecal samples

Alternations of contents of SCFAs which are fecal fermentation end products, indirectly express the gut microbiota composition. In the present study, The contents of SCFAs (including acetate acid, propionate acid and butyrate acid) in the faeces were determined to discuss the relationship between them and the gut microbiota of diabetes.

A fecal sample of 500 mg was accurately weighed and placed in a 15 ml centrifuge tube, and then 3 ml extract solvent (comprised of 0.1 mol/l oxalic acid, 40 mmol/l sodium azide and 0.1 mmol/l caproic acid) was added. The mixtures were shaken for 60 min, and then centrifuged at 14,000 g for 10 min. The supernatant obtained was filtered with 0.45 μm organic filter membrane (Navigator Lab instrument Co., Ltd, Tian Jing, China) for assay. In addition,
the standard solutions of acetate acid, propionate acid and butyrate acid in different concentrations were prepared in ultrapure water, with n-hexanoic acid as internal standard. All assays were performed on a Agilent GC 7890 Gas Chromatography System (Agilent Technologies). The separation was achieved on DB-WAXcolumn (30 m × 250 μm, 0.25 μm). The initial oven temperature was 120 °C and was kept there for 2.5 min, and then raised to 130 °C by 8 °C/min and held there for 4.0 min, then further increased to 210 °C by 30 °C/min, and finally held at 210 °C for 2 min. The temperature of FID and injection port were set at 250 °C and 200 °C, respectively. The injected sample volume was 1 μL. Flow rate of nitrogen, carrier gas was 3 ml/min with a 20:1 split ratio.

Statistical analysis

All data in this study are expressed in the form of mean ± SD. The data were evaluated by one-way analysis of variance (ANOVA). The Pearson’s correlation was conducted by SPSS statistics 19. Graphics presented using Origin 6.1. A 95% confidence interval (CI) was used as the threshold to identify potential outliers in all samples, OTUs clustering was analysed by the software system of TMEV Clustering.

Results

Chemical profiles of TSPK and PSPK

Steroidal saponins, constituted by 5 kinds of sapogenins such as dioxygenin and ruscogenen, displayed various biological activities of this medical plant. Dioxygenin was the major structure of steroidal saponin contained in TSPK (Yang et al., 2007; Yu et al., 2009a, b). In the meantime, polysaccharides accounted for another vital chemical type in P. kingianum. The content of total polysaccharides was quality control index for Polygonati Rhizoma, according to the Chinese Pharmacopoeia (2015 edition).

Therefore, determination of dioxygenin content in TSPK hydrolyzed sample and purity measurement of PSPK were the optimal solution to provide quantification information about TSPK and PSPK. Determination of dioxygenin of TSPK hydrolyzed by UPLC showed that the retention time of dioxygenin in hydrolyzed sample of total saponins was 21.288 min (RSD = 0.0089, the retention time of the standard of dioxygenin was 21138 min). This quantitation method of dioxygenin displayed good linearity within 0.025–0.500 mg/ml. The linear relationship of standard of dioxygenin was Y = 62,842.37 + 2.04 × 107X, the dioxygenin content in TSPK was 0.0295 mg/g. Polysaccharide purity in PSPK was measured by anthrone-sulfuric acid colorimetric methods. According to the linear relationship Y = 36.754X + 0.0541 of standard glucose, the final quality of the pure PSPK was 86.49%.

Measurement of body weight, insulin and blood glucose levels

At the end of this research, body weights in DM group and other treated group were lower than rats fed standard chow. Rats fed standard chow increased about 20.97% body weight. There is no significant differences in the changes of body weight between DM group and medication groups. (Fig. 2A)

At the end of this research, Met and TSPK.L treatments could significantly increase insulin levels by 76.25% and 103.59%, respectively (P < 0.01) (Fig. 2B). It indicated that Met and TSPK.L could partial recover the insulin secretion function of the beta cells. Correspondingly, the diabetic rats showed a significant increasing in the blood glucose level compared with the normal control group (P < 0.001). After 56 days continuously treatment of Met, TSPK (TSPK.L, TSPK.H) and PSPK, fasting blood glucose levels were significantly reduced by 42.99%, 29.28%, 24.23% and 48.40% respectively (P < 0.05, P < 0.01), when compared with the diabetic group (Fig. 2C).

TSPK and PSPK regulated gut microbial colony in the gut environment

The small intestinal contents of rats were cultivated for 24 h on blood agar plates to visually illustrate possible effects of TSPK and PSPK on DM rats. STZ injection and subsequent influences significantly reduced the diversity of bacterial species in intestinal of DM rats. Species diversities in TSPK.L and PSPK group were delightly improved to a level similar or even better than the control group (Fig. 3). It was suggested that 0.025 g/kg of TSPK and 0.1 g/kg of PSPK might be beneficial to intestinal ecological diversity.

Effects of TSPK and PSPK on LPS level

LPS, a cell-wall component of gram-negative bacteria, delivered to the liver via the portal vein in endotoxia. Such endotoxin production by gut microbiota in diabetes can cause chronic low-grade inflammation.

The level of LPS in DM group was not significantly changed compared to CON group (Fig. 4). However, the LPS levels in TSPK.L and PSPK groups remained at a lower level than DM rats. TSPK.L and PSPK could improve the intestinal species diversities without potential risk on LPS induced endotoxia.

TSPK and PSPK regulated the structural changes of gut microbiota

The relationships between the gut microbiota imbalance and diabetes pathogenesis were widely recognized (Qin et al., 2012). Specific variations in gut microbiota were observed in diabetes rats, such as higher abundance of Bacteroidetes and lower abundance of Firmicutes. It was considered as environmental factor for the development of diabetes (Noble et al., 2011). In this study, the microbial structural in different experiment groups were analyzed in different classification level.

Relative abundance in phylum level revealed that, the abundances of Bacteroidetes, Firmicutes and Proteobacteria in the CON group were 40.63%, 52.13%, 4.26% respectively. However, abundances of Bacteroidetes (63.36%), Proteobacteria (6.95%) increased in DM group, while the abundance of Firmicutes (29.46%) decreased. Met, TSPK and PSPK treatments could modulate the relative proportions of Bacteroidetes, Firmicutes and Proteobacteria (Fig. 5A). This might be related to the FBG reduction effects of TSPK and PSPK in diabetes.

Relative abundance of microbial species at Family level revealed that, relative abundance of Prevotellaceae, Bacteroidaceae (Bacteroides phylum) and Enterobacteriaceae (Proteobacteria phylum) in the diabetic group showed a high abundance (43.57%, 11.60% and 4.73%, respectively), compared to the normal rats (20.45%, 1.78% and 0.51%). Treated with TSPK.L and TSPK.H and PSPK significantly reduced the abundances of Prevotellaceae (30.05%, 30.21% and 31.15%), Bacteroidaceae (4.52%, 3.15% and 3.31%) and Enterobacteriaceae (0.63%, 0.63% and 1.41%). What’s more, compared with the DM group, TSPK.L and PSPK significantly increased the abundances of Ruminococcaceae (Firmicutes phylum) (26.63%; 23.15%), TSPK.H increased that of Veillonellaceae (Firmicutes phylum) to 37.59% (Fig. 5B).

In addition, we observed (Fig. 5C) that TSPK and PSPK inhibited the growth of Prevotella genus (Bacteroides phylum). Moreover, TSPK.L and PSPK significantly increase the abundances of Ruminococcus (Ruminococcaceae) (14.79% and 8.74%), and TSPK.H increased the abundance of Anaerovibrio (Veillonellaceae) (28.13%) significantly.
Correlation between 57 key OTUs and FBG, FINS, LPS and body weight

Pearson correlation analysis and cluster analysis were carried out based on clean data of OTUs (Operational Taxonomic Units).

By using Pearson correlation analysis, we identified 57 key variables, which were significantly altered after treated by TSPK and PSPK, correlated to the alternations of FBG, FINS, LPS and body weight (Fig. 6). Among them, Prevotellaceae (2 OTUs, belong to the Bacteroidetes), S24-7 (5 OTUs, belong to the Bacteroidetes), Lachnospiraceae (11 OTUs, belong to the Firmicutes) and Ruminococ-
TSPK and PSPK regulated SCFAs in faeces

Concentrations of SCFAs in faeces (acetate acid, propionate acid, butyrate acid), which were major microbial fermentation products of diets, were measured to display the possible regulation effects of TSPK and PSPK (Fig. 7).

Generally, TSPK.L and PSPK reduced the acetate, propionate and butyrate acids contents of these experimental rats (Fig. 7B, C, D). Therefore, the total SCFAs contents in the groups of TSPK.L and PSPK rats were significantly lower than the other group (Fig. 7A).

Among them, propionate and butyrate acids contents in DM group increased about 29.54% and 6.98% respectively, compared to the CON group. Treatment of TSPK.L and PSPK decreased the concentrations of propionate (19.30%, 36.26%) and butyrate acids (19.56%, 15.22%) respectively, related to the DM group. TSPK.L and PSPK significantly decreased the contents of SCFAs that may be related to the increased abundance of Ruminococcus. Previous studies (Cann et al., 2016; Chassard et al., 2012) pointed out that, Ruminococcus, as a kind of probiotics, had higher efficiency in the decomposition and utilization of fiber and carbohydrate in the diet.

However, TSPK.H increased that of propionic acid (81.11%) related to the DM group. This might be related to the increase of Anaerovibrio abundance in TSPK.H group. Anaerovibrio, also considered as probiotics, only decomposed limited carbohydrate (Prins et al., 1975).

TSPK.L and TSPK.H displayed discrepant effects on gut microbiota. Therefore, concentrations of propionic, acetic and succinic acids, end products of carbohydrate fermentation, were closely related to the changes of Ruminococcus and Anaerovibrio.

Fig. 3. Effects of TSPK and PSPK on microbial diversity of the colony in small intestinal tract
The small intestinal contents of rats were cultivated for 24 h on blood agar plates. The number of colonies was significantly lower in the DM group compared to the CON group. TSPK.L and PSPK increased about 370% and 70% colony forming units compared with DM group, which indicated a significant improvement on species diversity.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>BLANK</th>
<th>CON</th>
<th>DM</th>
<th>MET</th>
<th>TSPK.L</th>
<th>TSPK.H</th>
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<td>133</td>
<td>186</td>
<td>632</td>
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Fig. 4. TSPK and PSPK regulated enterogenous endotoxin.
The level of LPS in DM group was not significantly changed compare to CON group. TSPK.L and PSPK could improve the intestinal species diversities without potential risk on LPS induced endotoxemia.

The * indicates a significant difference compared with control group, * P < 0.05, ** P < 0.01, *** P < 0.001.

The # indicates a significant difference compared with model group, * P < 0.05, ** P < 0.01, *** P < 0.001.

group. However, treatment of TSPK.L and PSPK shortened the distance to the CON group. It suggested that the profile of gut microbiota in diabetic rats group were changed significantly compared to the normal rats. Met, TSPK.L and PSPK treatment could partially recover the gut microbiota equilibrium. The result was coincided with the results of colony in intestinal tract which cultivated for 24 h on blood agar plates (Fig. 3).

Discussions

It is now accepted that the acquisition and stability of gut microbiota are controlled and influenced by both environmental factors and host genetics. In turn, gut microbiota displayed feedback regulation on host health conditions, such as body weight, blood glucose and insulin sensitivity (Spor et al., 2011). Clearly, the modification of gut microbiota may trigger the other two and finally affect the body homoeostasis, collectively.

In our previous research (Lu et al., 2016), we found that TSPK could promote not only glycogenesis but also glucose utilization in peripheral tissues. However, some clues of gut microbiota regulation of TSPK were also provided. Therefore, we tried to determine the mechanism involved in the gut microbiota regulation of diabetic rats for P. kingianum.

The results of our study demonstrated that DM rats, induced by HFD feeding combined with low dose streptozotocin injection, were in hyperglycemia with impaired insulin secretion. Alterations in the composition of the gut microbiota were also observed in these DM rats. Increasing in the relative abundance of Bacteroidetes and Proteobacteria and decreasing in the relative abundance of Firmicutes were observed in DM rats.

Oral administrated with Met, TSPK and PSPK could alleviate hyperglycemia. In addition, the TSPK (TSPK.L, TSPK.H) and PSPK group increased Firmicutes abundance and reduced the abundance...
Fig. 5. TSPK and PSPK regulated the structural of gut microbiota. The microbial structure in different experiment groups were analysed in different species classification level of gut microbiota. The relative abundance of microbial species in Phylum (A), Family (B) and Genus (C) level in different groups were showed.
Fig. 6. Correlation between 57 key OTUs and diabetes test index
57 key variables were identified after Pearson correlation analyzed, which were significantly altered after treated by TSPK and PSPK, correlated to the alternations of FBG, FINS, LPS and body weight (A), clustering analysis of OTUS (B) and represented bacteria taxa information (genus, family and phylum) of 57 key OTUs (C) were shown. The symbol “+” shows that the OTUs correlated positively with FBG, weight, FINS, LPS in different groups, while, “−” show that the OTUs correlated negatively with FBG, weight, FINS, LPS in different groups.
of Bacteroidetes and Proteobacteria. These results suggested that the blood glucose regulation of TSPK and PSPK were closely related to the regulation of gut microbiota.

Earlier studies in humans, as well as in mice, reported that impaired glucose metabolism was associated with an altered ratio between the two major phyla in the human gut, Firmicutes and Bacteroidetes (Zhang et al., 2012; Li et al., 2008). In our research, TSPK and PSPK decreased the ratio of Bacteroidetes/Firmicutes and inhibited the abundance of Proteobacteria. The results of structural changes of gut microbiota and Pearson correlation showed that Firmicutes abundance was negatively correlated to FBG, however, positive correlated to body weight. Abundance increasing of Firmicutes and FBG decreasing were all observed after treatment of Met, TSPK and PSPK.

LPS, found in the outer membrane of Gram-negative bacteria, triggers the subclinical state of pro-inflammation, which is a characteristic feature of chronic low degree inflammation in type 2 diabetes. Our study showed that TSPK and PSPK treatments could decrease the contents of LPS. This indicated that TSPK, and PSPK may promote the growth of some beneficial bacteria, selectively.

Results from Pearson correlation indicated that most of Firmicutes, such as Lachnospiraceae and Ruminococcaceae, were negatively correlated with FBG, while positively correlated with body weight. Most of Bacteroidetes and Proteobacteria were positively correlated with LPS, but the Bifidobacterium and Ruminococcus were positively correlated with FINS. Related research found that Lachnospiraceae and Ruminococcaceae were the predominant acetogens in the enrichments of acetic acid (Gagen et al., 2015). In addition, The probiotic strain Bifidobacterium was predicted to be involved in the metabolism of dietary, plant-derived glycos, in particular starch and polysaccharides (Duranti et al., 2016).

In addition, the gut microbiota also influenced glucose and energy metabolism through the production of SCFAs. Butyrate, acetate, and propionate were produced through fermentation of complex polysaccharides by the colonic gut bacteria and enter the circulation. SCFAs not only acted as a source of energy in the body, but also widely distributed in the body’s organs from blood. The propionic acid mainly participated in hepatic glucose metabolism (Roy et al., 2006). Studies showed that (Bloemen et al., 2009), 70% of acetic acid in intestinal were uptaken by the liver, and involved in the synthesis of cholesterol, long chain fatty acid and glutamic acid, and 30% of them were metabolized by heart, kidney, etc. Butyric acid mainly involved in energy metabolism in the colon (Donohoe et al., 2011). Thus, SCFAs might influence host glucose

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**Fig. 7.** TSPK and PSPK regulated short-chain fatty acids in faeces

Contents of total short-chain fatty acids (A), acetate acid (B), propionate acid (C) and butyrate acid (D) in faeces were measured by gas chromatography. DM group significantly increased contents of propionate acid compared to normal group, however, administration of TSPK,L and PSPK could decrease the rise of propionate acid. To the contrary, TSPK,H raised in the contents of total SCFAs and propionate acid.

The * indicates a significant difference compared with control group, * P < 0.05, ** P < 0.01, *** P < 0.001.

The # indicates a significant difference compared with model group, * P < 0.05, ** P < 0.01, *** P < 0.001.

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TOTAL SCFCs | CON | DM | Met | TSPK,L | TSPK.H | PSPK
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metabolism through regulation of intestinal gluconeogenesis. TSPK and PSPK could also affect the SCFAs concentrations in DM rats.

Conclusion
In summary, TSPK and PSPK relieved hyperglycemic state in DM rats by the regulation of gut microbiota and subsequent SCFAs alteration. Interestingly, TSPK.L and PSPK increased the abundance of Ruminococcus, however, TSPK.H increased that of Anaerovibrio. Both Ruminococcus and Anaerovibrio were considered as probiotics. Thus, they showed discrepant regulation effects on the production of SCFAs. Related research are under further investigation in our follow-up work.

Conflict of interest
We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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