



Research article

Hypoglycemic mechanisms of *Polygonatum sibiricum* polysaccharide in db/db mice via regulation of glycolysis/gluconeogenesis pathway and alteration of gut microbiota

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ABSTRACT

Polygonatum rhizoma polysaccharide (PP) is a main ingredient of *Polygonatum rhizoma*, which is both food and traditional herbal medicine. In this study, we aimed to investigate the hypoglycemic effect of PP and the underlying mechanisms in db/db mice. Our finding showed that PP significantly ameliorates diabetic symptoms by reducing glucose levels in blood and urine and increasing insulin and leptin abundance in the serum. Histopathological examination revealed that PP improved the pathological state and increased hepatic glycogen storage in liver. Additionally, RT-qPCR results indicated that PP significantly down-regulated the expression of phosphoenolpyruvate carboxykinase 1. Furthermore, 16s rRNA sequencing results demonstrated that PP intervention resulted in an increase in beneficial bacteria genus and a reduction in harmful genus. Redundancy analysis revealed the correlation between intestinal flora and clinical factors. Taken together, these results suggest that PP has a significant hypoglycemic effect on type 2 diabetes (T2D) through up-regulating serum insulin and leptin, as well as hepatic glycogen storage, and down-regulating hepatic phosphoenolpyruvate carboxykinase 1 expression, as well as modulating gut microbiota composition. In conclusion, this study investigated the mechanisms of PP in the treatment of diabetes in db/db mice. To the best of our knowledge, this is the first study to explore the positive and negative correlations between gut microbiota and clinical factors, such as oxidative stress injury in liver and glucose related indicators in the blood.

1. Introduction

Diabetes is a chronic metabolic disease characterized by high levels of glucose in the blood, which can cause serious damage to the heart, blood vessels, eyes, kidneys, liver, brain and other organs [1]. Globally, there are over 400 million people with diabetes, and the disease is responsible for 1.5 million deaths per year [2]. Type 2 diabetes (T2D) accounts for over 90% of all diabetes cases [3].

Clinical data has shown that the quality and quantity of carbohydrates are important in the management of diabetes [4,5]. Polysaccharides derived from plants [6–16], animals [17], and fungi [18–20] have exhibited hypoglycemic activity and have also been shown to ameliorate complications associated with diabetes in organs such as the kidneys [20,21], liver [22,23], pancreatic islets [24,

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25], and heart [26]. The mechanisms underlying these effects mainly involve increased glycogen storage and reduced gluconeogenesis, as well as targeting inflammation and alteration of gut microbiota [20–26].

Polygonatum sibiricum Red., a plant species in the family Liliaceae, is native to Asia, specifically to regions such as China, Korea, Mongolia and Russia. The rhizome of *Polygonatum sibiricum Red* has a sweet and refreshing taste and contains a variety of nutrients, including carbohydrates, fats, proteins, starch, carotene, and vitamins. It can be eaten raw or stewed and is both nourishing and good for physical fitness. In traditional Chinese medicine, *Polygonati rhizoma* is the dried rhizome of the plant *Polygonatum sibiricum Red.*, commonly used as a medicinal herb to tonify qi and nourish yin, strengthen the spleen, moisten the lungs, and nourish the kidneys. It is often prepared as a decoction or tea. *Polygonatum rhizoma* polysaccharide (PP) is one of the main components of *Polygonatum sibiricum*, which is both a medicine used to treat diabetes and a kind of food. PP was reported to have many biological activities and pharmacological applications, including antioxidant, anti-aging, anti-fatigue, immunity enhancement, antibacterial, anti-inflammatory, hypolipidemic and antiatherosclerotic, anti-osteoporosis, liver protection, and anti-cancer effects, as well as a hypoglycemic function in Alloxan- or Streptozocin-induced diabetes models [27]. However, its mechanism of hypoglycemic effect is still unclear, and the effect of PP on natural diabetes remains unknown.

To elucidate the hypoglycemic mechanisms of PP in natural T2D, this study was conducted using db/db mice. The findings will provide valuable data for enhancing scientific understanding and functional applications of PP.

2. Results and discussions

2.1. Effects of PP on body weight and organ index

Following six weeks of treatment, the mice were sacrificed and their body weight and liver index were shown in Table 1. Both db/db mice and normal control mice showed an increase in body weight during the experiments. However, body weight and liver index were significantly higher in the diabetic model (DM) group compared with the normal control (NC) group. There was no significant difference observed in body weight or liver index between the PP group and DM group. These results suggest that PP does not have toxicity to db/db mice.

2.2. Effect of PP on blood glucose, serum glucose, urinary glucose, oral glucose tolerance, serum insulin, and glycated serum protein (GSP)

The hypoglycemic effects of PP on T2D were investigated in db/db mice. Throughout the experiment, blood glucose levels were measured from the tail vein once a week to illustrate the dynamic hypoglycemic effects of PP. Compared to the DM group, the PP group showed a decreasing trend in blood glucose levels from the second to the fifth week, and the difference became significant in the sixth week ($p < 0.05$, Fig. 1A). These results confirmed that PP has a hypoglycemic effect in naturally occurring T2D in db/db mice. Additionally, glucose levels from femoral artery serum were measured at the end of the experiment, and the results showed a significant decrease in the PP group compared to the DM group ($p < 0.05$, Fig. 1B), further supporting that PP can reduce blood glucose levels in db/db mice.

Moreover, urinary glucose levels were also evaluated. High urinary glucose level is one of the main symptoms of diabetes [28]. The results showed that urinary glucose levels were significantly decreased in the PP group compared to the MD group ($p < 0.05$, Fig. 1C), suggesting that PP could not only reduce glucose level in the blood but also in urine. Oral glucose tolerance is a criterion to diagnose diabetes [29]. In this study, oral glucose tolerance was tested and the area under the blood glucose curve (AUC) was calculated accordingly. The AUC was significantly reduced in the PP group compared to the DM group ($p < 0.05$, Fig. 1D), providing further evidence of the hypoglycemic effect of PP in db/db mice.

To investigate the long-term hypoglycemic effect of PP, a more stable biomarker, GSP was tested. GSP reflects mean blood glucose levels over a period of 2–3 weeks [30]. The results showed that the GSP level was significantly decreased in the PP group compared to the DM group ($p < 0.05$, Fig. 1E), indicating that PP had a long-term hypoglycemic effect in db/db mice.

T2D is characterized by impaired insulin secretion and action [31]. The abundance of insulin was significantly lower in the DM group than in the NC group, and significantly increased in the PP group compared to the DM group ($p < 0.05$, Fig. 1F). Furthermore, the insulin level in the PP group showed no significant difference compared to the NC group ($p > 0.05$), indicating that PP could restore the low insulin level in db/db mice to normal level.

All the results presented above provide solid evidences that PP has a significant hypoglycemic effect in db/db mice.

Table 1
Effects of PP on body weight and liver index in db/db mice.

Groups	Initial BW(g)	Final BW (g)	Liver index (g/100 g BW)
NC	20.8 ± 0.6 b	26.2 ± 1.5 b	3.97 ± 0.24 b
DM	40.8 ± 4.6 a	48.4 ± 5.1 a	4.61 ± 0.64 a
PP	40.2 ± 1.9 a	51.2 ± 3.1 a	5.15 ± 0.56 a

Values represent means ± standard deviations. Statistical analysis was performed using SPSS statistics 21.0, and the ANOVA test followed by the LSD and SNK test was conducted. Different letters (a, b) in the same column indicate a significant difference among interventions ($p < 0.05$) NC: m/m mice + water; DM: db/db mice + water; PP: db/db mice + polygonatum polysaccharide (1.0 g/kg-d); BW: body weight.

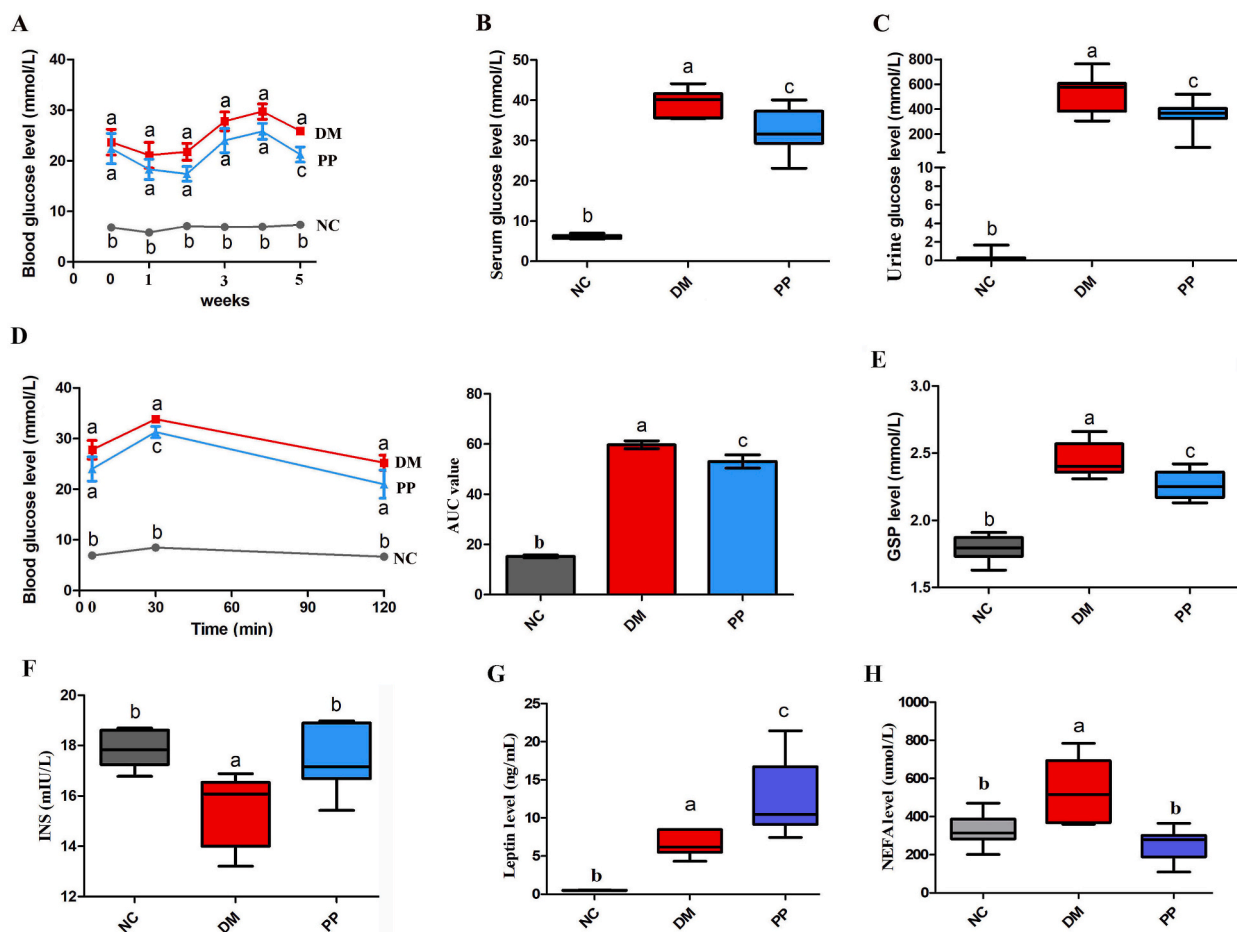


Fig. 1. Hypoglycemic effects of PP in db/db mice. A, Blood glucose level; B, Serum glucose level; C, Glucose level in urine; D, Oral glucose tolerance and AUC; E, GSP level; F, INS level; G, Leptin level; H, NEFA level. Values represent means \pm standard deviations. Statistical analysis was performed using SPSS statistics 21.0, and the ANOVA test followed by the LSD and SNK test was conducted. Different letters (a, b, c) in the same figure indicate a significant difference among groups ($p < 0.05$). NC: m/m mice + water; DM: db/db mice + water; PP: db/db mice + PP.

2.3. Effect of PP on leptin and nonesterified fatty acid (NEFA)

Leptin has been shown to suppress diabetic hyperglucagonemia [32] through its interaction with multiple factors [33–35]. In this study, we evaluated the effect of PP on serum leptin abundance. Our results indicated that leptin was significantly more highly expressed in the DM group than the NC group ($p < 0.05$, Fig. 1G). This is consistent with previous reports that have found a positive genetic correlation between leptin levels and with T2D [36]. Conversely, leptin was more highly expressed in the PP group than DM group ($p < 0.05$, Fig. 1G). Previous studies have shown that leptin acts upstream in the regulation of insulin secretion [37]. Here, the changes in leptin levels due to PP treatment showed similar trends to the results of insulin shown in Fig. 1F, indicating a positive interaction between leptin and insulin levels [37]. These results suggest that PP might down-regulate glucose levels by stimulating the expression level and interaction of leptin and insulin in db/db mice. However, since the db/db mouse is a leptin receptor mutant mouse, further clarification is required to understand how leptin effect insulin secretion in leptin receptor-independent pathway.

NEFA has been shown to induce insulin resistance and impair β -cell function, making it a likely culprit [38]. It is increased in T2D, and carefully adjusting its levels has been inversely associated with diabetes risk [39,40]. In this study, NEFA levels were determined, and the results showed that they were significantly more highly expressed in the DM group than in the NC group, but were significantly reduced in the PP group compared to the DM group (Fig. 1H). The downregulation of NEFA by PP might contribute to its hypolipidemic and hypoglycemic effect in db/db mice.

2.4. Effect of PP on pathology, glycogen level, oxidative stress and genes related to glucose metabolism in liver

The liver is a central insulin target organ and plays a unique role in maintaining the glucose homeostasis of the whole body. A healthy and intact cellular structure in the liver is essential to improve diabetes. Therefore, we observed the pathology, glycogen level,

oxidative stress, and genes related to glucose metabolism in the liver.

Firstly, we investigated the protective effect of PP on liver injury induced by diabetes using representative images of liver sections stained with hematoxylin and eosin (HE) and periodic acid-Schiff reagent (PAS). As shown in Fig. 2A and B, the hepatocyte morphology was regular and well-arranged in the NC group, while there was lipid accumulation and vacuolation in a large proportion of liver cells in the DM group. This is consistent with clinical practice, as T2D patients often show vacuolated nuclei and acidophilic bodies in liver biopsy specimens [41]. As anticipated, there was less lipid accumulation and fewer cells containing vacuoles in the PP

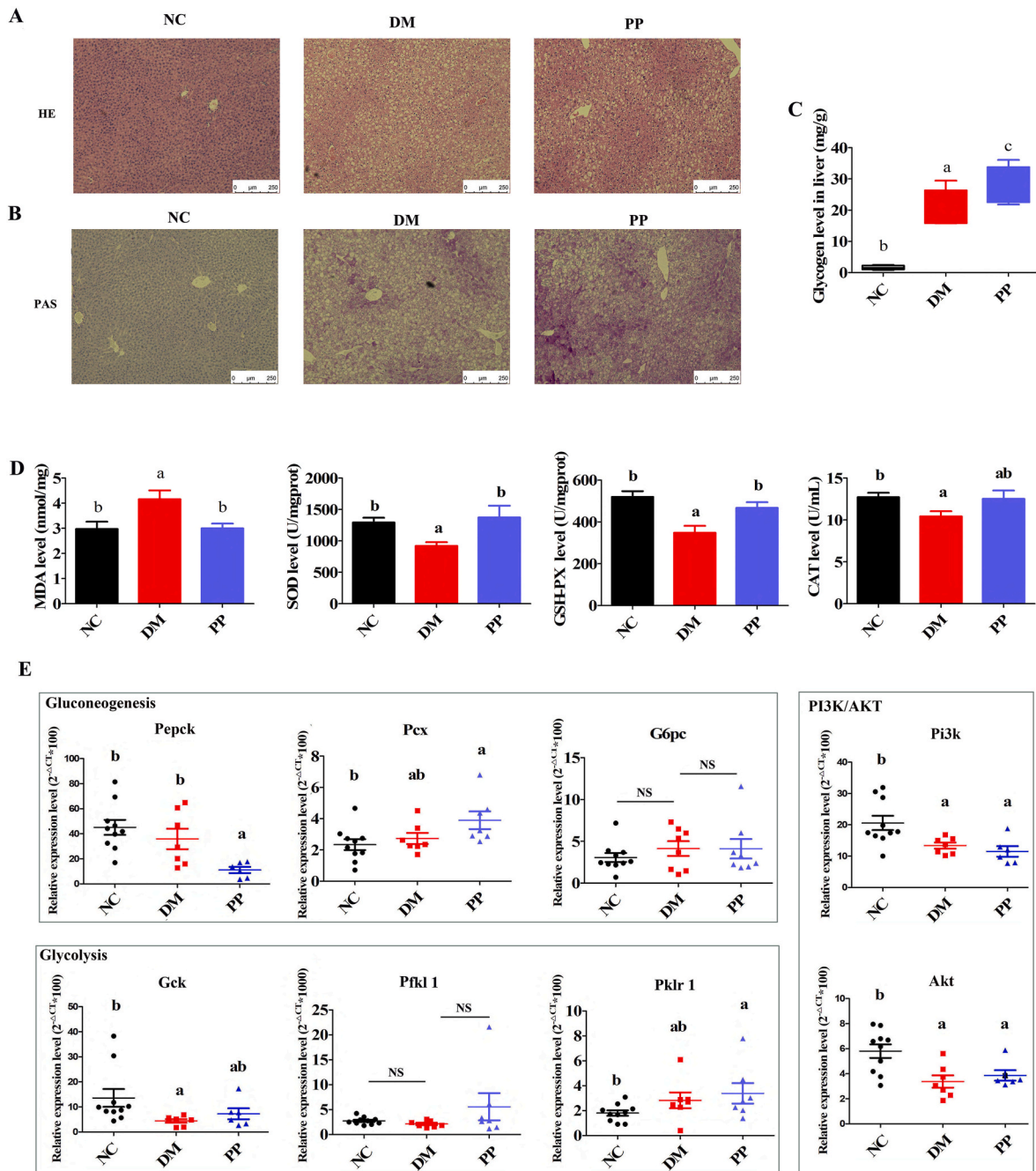


Fig. 2. Effect of PP on oxidative stress, glycogen and expression level of relative genes in liver of db/db mice. A, HE staining (10 ×); B, PAS staining (10 ×); C, Glycogen abundance; D, Oxidative stress; E, The mRNA level and/or protein level of genes in Glycolysis/Gluconeogenesis pathway or PI3K/AKT pathway. Values represent means ± standard deviations. Statistical analysis was performed using SPSS statistics 21.0, and the ANOVA test followed by the LSD and SNK test was conducted. Different letters (a, b, c) in the same figure indicate a significant difference among groups ($p < 0.05$). NS indicate no significant. NC: m/m mice + water; DM: db/db mice + water; PP: db/db mice + PP.

group compared to the DM group, suggesting that the severe liver injury induced by diabetes was ameliorated by PP intervention.

Secondly, we observed the glycogen content using PAS staining among the three groups. As shown in Fig. 2B, the glycogen content was increased in the DM group compared to the NC group, which was in accordance with a previous report showing that db/db mice exhibit higher glycogen content in the liver [9]. PP intervention increased the glycogen content in db/db mice (Fig. 2B). The increase in hepatic glycogen synthesis is beneficial in reducing body glucose levels due to the consumption of glucose. To confirm these observations, glycogen abundance was further quantified by ELISA. As shown in Fig. 2C, the glycogen level was significantly higher in the PP group than in the DM group, which further supported the results observed from the pathological section. These findings were consistent with a previous report showing that the glycogen level is increased along with the improvement of diabetes in db/db mice [9].

Thirdly, to further confirm the liver protective function of PP in diabetic db/db mice, we tested the oxidative stress condition among groups by detecting the level of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in the liver. Experimental and clinical evidence has shown that diabetes is closely associated with oxidative stress [42]. Our results in Fig. 2D showed that the MDA was increased significantly ($p < 0.05$), while SOD, CAT and GSH-Px were significantly decreased ($p < 0.05$) in the DM group compared to the NC group, indicating that the db/db mouse experienced oxidative stress in the liver. After PP intervention, MDA was significantly down-regulated ($p < 0.05$) and SOD and GSH-Px were significantly up-regulated ($p < 0.05$) compared to the DM group. No significant difference was found between the NC group and DM group for above three indicators ($p > 0.05$). CAT was also up-regulated to a normal level, although the difference between the DM group and PP group was not significant. These results indicated that PP could reduce the amount of MDA in diabetic db/db mice, and increase the activities of SOD, GSH-Px, and CAT enzymes, thus significantly relieving oxidative stress in the liver of diabetic db/db mice.

Fourthly, we investigated the expression levels of key genes involved in the gluconeogenesis pathway, glycolysis pathway and PI3K/Akt pathway in liver. The liver plays a critical role in maintaining short- and long-term glucose homeostasis by regulating glucose production and storage through the expression of numerous genes [43], like genes in the Glycolysis/Gluconeogenesis pathway and PI3K/AKT pathway [44]. We examined genes in the Glycolysis/Gluconeogenesis pathway and PI3K/AKT pathway and found no significant differences in *Pepck*, *Pcx*, and *G6pc* expression levels between the DM group and NC group ($p > 0.05$, Fig. 2E). However *Pepck* expression was significantly down-regulated ($p < 0.05$) in the PP group compared to the DM group, while there was no significant difference in *Pcx* and *G6pc* expression levels between the PP and DM groups ($p > 0.05$). These findings suggest that PP inhibits gluconeogenesis and reduces blood glucose by decreasing *Pepck* expression rather than *Pcx* or *G6pc*. *Pepck* catalyzes one of the rate-limiting steps of hepatic gluconeogenesis, and insulin can suppress gluconeogenesis by inhibiting *Pepck* expression [43]. In the PP group, *Pepck* expression was significantly down-regulated compared to the DM group (Fig. 2E), while insulin levels were significantly

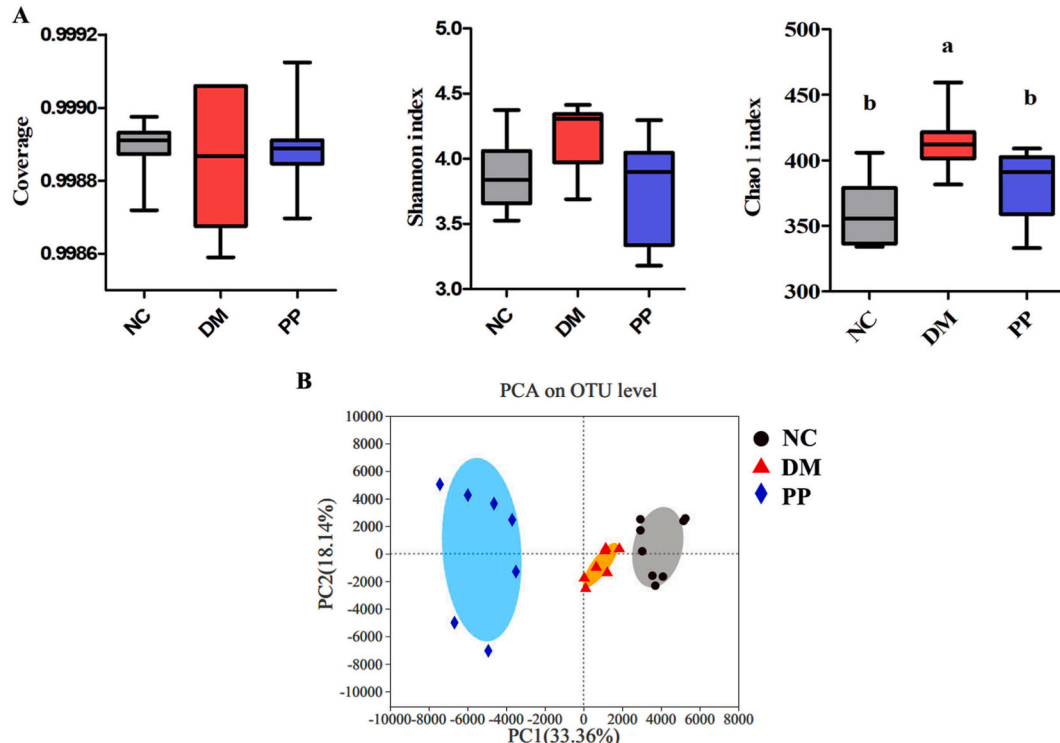
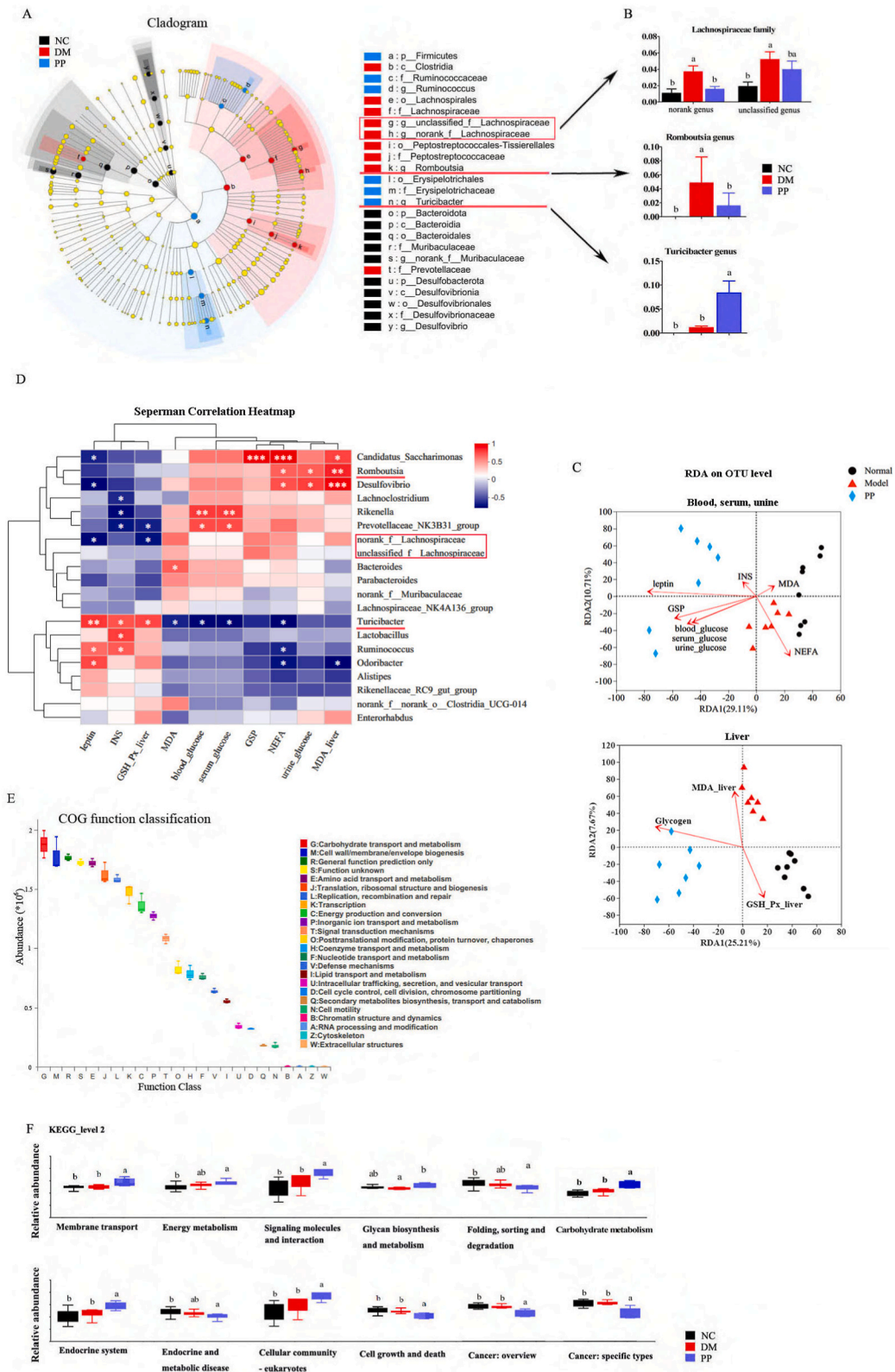


Fig. 3. The alpha diversity analysis (A) and PCA analysis (B) Values represent means \pm standard deviations. Statistical analysis was performed using SPSS statistics 21.0, and the ANOVA test followed by the LSD and SNK test was conducted. Different letters (a, b) in the same figure indicate a significant difference among groups ($p < 0.05$). NC: m/m mice + water; DM: db/db mice + water; PP: db/db mice + PP.



(caption on next page)

Fig. 4. (A) LefSe analysis (LDA threshold was set to 4) for the characteristics of flora among groups in mice. Different colors represent different groups. Notes that were colored indicated characteristic flora in the group, and the flora name was listed on the right of the graph. (B) The different relative abundance of flora among groups. (C) RDA analysis (D) Spearman correlation heatmap between clinical factors and flora genus. (E) The COG function classification predicted by PRCRUST. (F) The KEGG pathway abundance predicted by PRCRUST. Values represent means \pm standard deviations. Statistical analysis was performed using SPSS statistics 21.0, and the ANOVA test followed by the LSD and SNK test was conducted. Different letters (a, b) indicate a significant difference among groups ($p < 0.05$) in (B) and (F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NC: m/m mice + water; DM: db/db mice + water; PP: db/db mice + PP.

increased (Fig. 11). These results suggested that PP may prevent gluconeogenesis by stimulating insulin to inhibit *Pepck* expression in the liver, which can lead to lower glucose levels. The key genes in the glycolysis pathway include *Gck*, *Pfk* and *Pklr*, and we found that PP could increase the expression levels of all three genes to some extent compared to the DM group, although the changes were not significant ($p > 0.05$, Fig. 2E). The hepatic *Gck* plays a crucial role in whole-body glucose homeostasis by catalyzing the phosphorylation of glucose, and activators of *Gck* may be potential diabetes therapy [45]. The *Gck* expression was significantly decreased in db/db mice compared with normal mice ($p < 0.05$, Fig. 2E), which is consistent with previous reports [9]. Here, the *Gck* levels in the PP group did not differ significantly compared with either the NC group or the DM group, suggesting that *Gck* may be a potential target of PP for hypoglycemia therapy. The PI3K/Akt pathway is involved in glucose metabolism, and its activation can ameliorate hyperglycemia in diabetic mice [44]. We found both Pi3k and Akt were suppressed in db/db mice (Fig. 2E), consistent with previous studies [46]. However, neither Pi3k nor Akt were activated in the PP group compared to the DM group, although Akt had an upward trend (Fig. 2E). These results suggested that PI3K/Akt pathway may not be the main pathway for PP to reduce glucose level.

2.5. Effect of PP on gut microbiota

The gut microbiota, also known as the second genome of the host, has been reported to play an important role in T2D [47]. To investigate the regulation of PP on gut microbiota in diabetic db/db mice, 16s rRNA sequencing was performed. The alpha diversity indexes (Fig. 3A) and beta diversity index (Fig. 3B) at the OUT level were analyzed. The community coverage of all three groups was over 99.86%, and no significant differences were observed among the groups ($p > 0.05$, Fig. 3A). However, compared with the DM group, the community diversity represented by the Shannon index in the NC group and PP group showed a lower expression trend ($p > 0.05$, Fig. 3A). In contrast, the community richness represented by Chao 1 index in the NC group and PP group significantly decreased ($p < 0.05$, Fig. 3A), indicating that community richness in diabetic db/db mice was significantly increased, and PP intervention could significantly down-regulate it to normal levels. Principal Component Analysis (PCA) revealed obvious differences in bacterial composition among groups (Fig. 3B). These results suggested that the gut microbiota might be a target of PP for its hypoglycemic effect in diabetic db/db mice.

To reveal the unique characteristics of flora among groups, we conducted LefSe analysis (LDA threshold was set to 4) as depicted in Fig. 4A. In the DM group, four characteristic floras were identified, including two unknown genera in the Lachnospiraceae family of Lachnospirales order of Clostridia class, *Romboutsia* genus in Peptostreptococcaceae family of Peptostreptococcales-Tissierellales order of Clostridia class, and Prevotellaceae family (Fig. 4A and B). In the PP group, the characteristic floras were *Turicibacter* genus in the Erysipelotrichaceae family of Erysipelotrichales order of Firmicutes phylum, and *Ruminococcus* genus in the Ruminococcaceae family (Fig. 4A and B). Previous research has shown that the *Lachnospiraceae* genus bacterium induced significant increases in blood glucose levels in obese mice [48] and had a high relative abundance in diabetes, but was reduced during the improvement of diabetes [49,50]. *Romboutsia* was found to be markedly increased in T2D and decreased after treatment [10,51]. However, the *Turicibacter* genus was enriched in T2D and elevated during the improvement of T2D [52] and type 1 diabetes [53]. The *Ruminococcus* genus is associated with the progression of diabetes [54], while the enrichment of the Ruminococcaceae family is related to amelioration of inflammation in T2D rats [55]. PP reduced the abundance of two genera in the Lachnospiraceae family and the *Romboutsia* genus to a normal level but increased the abundance of *Turicibacter* genus and *Ruminococcus* genus in db/db diabetic mice. This finding suggested that of the hypoglycemic effect of PP may be due, in part, to these changes in gut microbiota. However, further experiments using germ-free mice are necessary to establish whether the gut microbiota play an essential role in the pharmacological effect of PP.

Table 2
RDA analysis data.

Clinical factor	Sample	RDA 1	RDA 2	R2	P values
Glucose level	tail vein blood	-0.9484	-0.3172	0.4056	0.01
	serum	-0.9484	-0.3172	0.4056	0.01
	urine	-0.894	-0.448	0.2892	0.039
GSP	serum	-0.8995	-0.4369	0.3325	0.022
INS	serum	-0.6113	0.7914	0.0268	0.816
Leptin	serum	-0.9994	0.0346	0.6291	0.001
NEFA	serum	0.4139	-0.9103	0.4171	0.007
MDA	serum	0.826	0.5637	0.0258	0.81
Glycogen	liver	-0.9507	0.3102	0.8261	0.001
MDA	Liver	-0.1971	0.9804	0.5029	0.002
GSH-Px	liver	0.4	-0.9165	0.4623	0.001

The correlation between intestinal flora and clinical factors was explored using redundancy analysis (RDA). The results indicated a significant correlation between clinical factors such as blood, serum, urinary glucose levels, serum GSP, leptin, and NEFA abundance, as well as liver glycogen, MDA, GSH-Px with gut microbiota composition ($p < 0.05$, Fig. 4C and Table 2). To further analyze the relationship between clinical factors and gut microbiota in diabetic db/db mice and PP intervention, a correlation heatmap was generated. The heatmap revealed that each clinical factor had an impact on one or more flora genera (Fig. 4D). For instance, the norank genus in the Lachnospiraceae family was negatively correlated with serum leptin and GSH-Px in the liver, inferring that it might be the target of PP to up-regulate serum leptin and hepatic GSH-Px in db/db mice. Additionally, the *Romboutsia* genus was positively correlated with serum NEFA, urinary glucose level, and liver MDA abundance, suggesting that it might play a role in increasing these clinical factors in the DM group. However, PP significantly down-regulate the *Romboutsia* genus (Fig. 4D) and all three factors (Fig. 1C, H and Fig. 3D), indicating that the *Romboutsia* genus might be the target of PP in lowering the excessively high indicators like serum NEFA, urinary glucose level and liver MDA in diabetic db/db mice. Furthermore, the *Turicibacter* genus was positively correlated with serum INS/leptin and hepatic GSH-Px but negatively correlated with blood/serum glucose and serum MDA/NEFA. Given that *Turicibacter* genus was a marker flora in the PP group, it was speculated that PP might be the prebiotics of *Turicibacter* genus. PP might play multiple roles, such as up-regulating serum INS/leptin and hepatic GSH-Px abundance and down-regulating blood/serum glucose level and serum MDA/NEFA content by raising the abundance of *Turicibacter* genus, thus ameliorating T2D in db/db mice.

To analyze the functions of gut microbiota, we utilized the Clusters of Orthologous Groups (COG) function classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) function predicted by PICRUSt. The results indicated that the main COG function was related to carbohydrate transport and metabolism, cell wall/membrane/envelope biogenesis, amino acid transport and metabolism, energy production and conversion (Fig. 4E). On the other hand, the KEGG pathway on level 2 showed that PP stimulated the abundance of signal pathways like carbohydrate metabolism, membrane transport, energy metabolism, signaling molecules and interaction, glycan biosynthesis and metabolism, endocrine system and cellular community eukaryotes, while inhibiting endocrine and metabolic disease, cell growth and death, cancer: overview, cancer: specific types and folding sorting and degradation pathway (Fig. 4F). Most of the above pathways are involved in diabetes, where carbohydrate metabolism [56,57], energy metabolism [58,59], membrane transport [60,61], and glycan biosynthesis and metabolism [62] are commonly affected. The carbohydrate metabolism in level 2 pathway contains several level 3 pathways, such as glycolysis/gluconeogenesis, fructose and mannose metabolism, as well as starch and sucrose metabolism pathways which were activated by PP (Fig. 4F). These three pathways directly influence the glucose levels, and the activation of the three signal pathways by PP was consistent with the results of gene detection as shown in Fig. 2E. Therefore, PP might improve diabetes in db/db mice by modulating all pathways mentioned above. Additionally, extensive evidence suggests that diabetes raised the risk of multiple cancers and involves poorer prognosis following a cancer diagnosis [63]. Hence, PP may prevent the development of cancer by the constraining cancer-related signaling pathways in diabetic db/db mice. These results require further experimental confirmation.

3. Conclusions

In summary, our study found that PP significantly reduced the glucose levels in tail vein blood, abdominal aorta serum, and urine of genotype T2D in db/db mice, indicating its effectiveness in treating diabetes. Furthermore, PP improved several symptoms of diabetes, such as lowering long- and short-term blood glucose levels, increasing insulin and leptin levels, and decreasing NEFA and MDA levels in serum. Additionally, PP demonstrated a liver-protective function in diabetic db/db mice by releasing oxidative stress in the liver. PP also shown to increase hepatic glycogen abundance and regulate the expression of genes (*Pepck*, *Pcx*, *Gck*, *Pklr 1*) involved in glucose storage and utilization in the liver. The results of 16s rRNA sequencing indicated that PP intervention increased the beneficial bacteria genus (*Turicibacter* genus) and reduced harmful bacteria genus (two genera in the Lachnospiraceae family and *Romboutsia*). This study is the first to investigate the mechanisms of PP in treatment of diabetes in db/db mice by analyzing the positive and negative correlations between gut microbiota and clinical factors such as oxidative stress injury in liver or glucose-related indicators in the blood. Overall, this study reported on the hypoglycemic effect and underlying mechanisms of PP in improving T2D through multipath including gut microbiota. These findings could provide a scientific basis for the application of PP as a natural anti-diabetic drug or function food due to its multi-target hypoglycemic effect.

4. Materials and methods

4.1. Animals and experimental design

Specific pathogen-free male db/db mice (8 weeks old, 35–40 g) and their age-matched non-diabetic m/m mice (18–20 g) were purchased from Changzhou Cavens experimental animal Co. Ltd (Changzhou, China). The mice were housed in specific pathogen barrier facilities with standard temperature and humidity and a 12 h light/dark cycle. Following a one-week acclimation for period, the db/db mice were randomly divided into the PP group and DM group ($n = 7$). The m/m mice were assigned to NC group ($n = 10$). The mice in the PP group were intragastric gavage with 1.0 g/kg BW/day PP (Shanghai Yuanye Bio-Technology Co. Ltd, Shanghai, China; the polysaccharide proportion of 9.0 kDa was 80.6%), and mice in DM or NC group were intragastric gavage with equal volumes of clean water. The treatment lasted for 6 weeks. All experimental protocols were approved by the Laboratory Animal Welfare Ethics Committee of the Zhejiang Academy of Traditional Chinese Medicine (Approval No: 2021–035).

4.2. Measurements of body weight and liver index

The body weight of each mouse was recorded weekly. After 6 weeks of treatment, all mice were anesthetized with pentobarbital sodium (50 mg/kg), and the livers were dissected and weighed. The organ index was calculated according to the formula: organ index = organ weight (g)/body weight (100 g).

4.3. Measurements of blood glucose, oral glucose tolerance, urinary glucose, GSP, and serum insulin

The blood glucose of each mouse was measured weekly via the tail vein. At the end of the experiment, the oral glucose tolerance of each mouse was assessed via the tail vein. All blood glucose levels via the tail vein were determined using a blood glucose test strip (ACON Biotech (Hangzhou) Co. Ltd, Hangzhou, China). Overnight urine samples were collected from each mouse in the metabolic cage, and urinary glucose levels were measured using a glucose determination kit (Shanghai Rongsheng Biotech Co. Ltd, Shanghai, China) in the sixth week.

After anesthetized, blood samples from the femoral artery were collected and placed at 4 °C for 2 h, and then centrifuged at 3,000 rpm for 10 min to obtain the serum. The GSP level was determined using a glycosylated serum protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The insulin level was determined using an ELISA assay kit (CUSABIO BIOTECH CO. LTD, Wuhan, China).

4.4. Measurements of leptin and NEFA level

The leptin was determined using an ELISA kit (CUSABIO BIOTECH CO. LTD, Wuhan, China). The NEFA was determined according to corresponding kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

4.5. Measurements of oxidative stress in the liver

The oxidative stress in serum and liver was evaluated through the level of MDA, CAT, SOD, and GSH-Px according to instructions of each assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The detection methods and commercial kit were Malondialdehyde (MDA) assay kit (TBA method) for MDA, CATalase (CAT) assay kit (visible light) for CAT, Total Superoxide Dismutase (T-SOD) assay kit (hydroxylamine method) for SOD, Glutathion Peroxidase (GSH-Px) assay kit (Colorimetric method) for GSH-Px, respectively.

4.6. Determination of hepatic histopathological and glycogen

Each liver was fixed in 4% (w/v) paraformaldehyde, embedded in paraffin, and sectioned to 4 μm. The sections were then stained with HE and PAS to evaluate the pathology and glycogen content. The slides were observed using a LEICA DFC 7000 T microscope. The hepatic glycogen was evaluated with a commercial kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

4.7. Detection of gene expression in liver

The relative mRNA expression level of *Pi3k*, *Akt*, *Gck*, *pPepck*, *Pcx*, *G6pc*, *Pfkl*, and *Pklr* were quantitated via quantitative real-time PCR using published method [64]. The primers were listed in Table 3. The endogenous control gene was β-actin. The relative

Table 3
Primer sequences.

Gene	Primer direction	Sequences
<i>Pi3k</i>	Forward	CCCCTACTGTAGCCAACAAC
	Reverse	CGTACCAAAAGGTCCCATCA
<i>Akt</i>	Forward	ATGAACGACGTAGCCATTGTG
	Reverse	TTGTAGCCAATAAAGGTGCCAT
<i>Gck</i>	Forward	TGAGCCGGATGCAGAAGGA
	Reverse	GCAACATCTTTACACTGGCCT
<i>Pepck</i>	Forward	ATGGGGTGTITGTAGGAGCA
	Reverse	CCGAAGTTGTAGCCGAAGAA
<i>Pcx</i>	Forward	GCAAGATGGGAGACAAGGTGGAAG
	Reverse	GAAGCCGTAGGTGTTGGAGAACTC
<i>G6pc</i>	Forward	TCCGTGCCATAATAAAGCAGT
	Reverse	TGGCTTTTCTTCTCGAAAG
<i>Pfkl</i>	Forward	CGCATCAAGCAGTCAGCCTCAG
	Reverse	ACAGTAGCCAGGTAGCCACAGTAG
<i>Pklr</i>	Forward	GGCTCAGAAGATGATGATTGGA
	Reverse	ATCGCATGTTGCATCTTTACAG
<i>β-actin</i>	Forward	GTGACGTTGACATCCGTAAGA
	Reverse	GCCGGACTCATCGTACTCC

expression levels were calculated using the $2^{-\Delta\text{Ct}}$ method [65].

4.8. Analyses of gut microbiota

The bacterial DNA from ileocecal part content was extracted using the E. Z. N. ATM Mag-Bind Soil DNA kit. Then the V3 – V4 regions of the 16s rRNA gene were sequenced with amplification primers 338 F and 806 R on the Illumina MiSeq sequencing platform. The RDA was performed using the RDA analysis in the vegan package of R language (version 3.3.1).

4.9. Statistical analysis

Data were presented as mean \pm standard deviations. The difference analysis was calculated using a one-way analysis of variance (ANOVA) test followed by LSD and SNK test using SPSS statistics 21.0. Differences were considered statistically significant at $p < 0.05$.

Author contribution statement

Guan-Hai Dai; Xuan Chen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ye-ling Tong; Si-si Chen; Xi-yu Mei; Qing-yun Zhou: Performed the experiments.

Ze-ming Ren: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

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