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Walnut green husk polyphenols ameliorate type 2 diabetes in C57BL/6 mice by the gut microbiota and IRS/PI3K/AKT signaling pathway

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ABSTRACT: Walnut green husk, a byproduct of walnut processing, is rich in polyphenols with potential antidiabetic effects. However, the antidiabetic activity of walnut green husk polyphenols (WGHP) *in vivo* has not been reported and their mechanism of action is still unclear. This study investigated its impact on type 2 diabetes in mice induced by a high-fat diet and streptozotocin. We found that walnut green husk polyphenols improved glycolipid metabolism, reduced inflammation and oxidative stress, and protected the pancreas and liver. WGHP also reshaped the gut microbiota, lowering the Firmicutes/Bacteroidota ratio and boosting beneficial bacteria. Network pharmacology indicated that WGHP's effects are mediated by the PI3K/AKT signaling pathway, which was confirmed by increased protein expression of p-IRS-1, PI3K, p-AKT, and GLUT4 in the liver. In conclusion, these results indicate that WGHP ameliorates T2DM by modulating gut microbiota and the IRS/PI3K/AKT pathway, providing a theoretical basis for the in-depth utilization of walnut green husk.

Key words: Walnut green husk polyphenols, T2DM, network pharmacology, gut microbiota, IRS/PI3K/AKT pathway

1. Introduction

Type 2 diabetes mellitus (T2DM) is a non-autoimmune metabolic disease that accounts for 90% of all cases of diabetes mellitus and is characterized by reduced insulin sensitivity or a lack of insulin signaling in the liver, skeletal muscle or fat tissue[1]. Studies have shown that dysregulation of adipokines, immune dysregulation, inflammatory effects, and abnormalities of the gut microbiota have become important pathophysiological factors in the pathogenesis of T2DM[2]. Currently, the drugs used to treat T2DM caused many serious side effects, such as weight gain, hypoglycaemia, worsening heart failure, osteoporosis, and an increased risk of bladder cancer[3-4]. Therefore, an alternative is pivotal for healthier medications or dietary

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supplements to improve T2DM. Moreover, bioactive substances from natural plants or food sources with medicinal properties are promising candidates due to their sustainability.

Walnut green husk is a layer of green, thick husk outside the walnut and a by-product during walnut processing, which is often discarded as waste, causing an environmental burden[5]. It is known that walnut green husk is rich in polyphenols, flavonoids and polysaccharides, as well as other bioactive substances, with antibacterial, antioxidant and hypolipidemic and other physiological activities[6]. Among them, walnut green husk polyphenols are ideal candidates for functional food and drug development due to their rich content and various biological activities. It has been shown that catechins and quercetin, which are polyphenols of walnut green husk, can exert hypoglycaemic effects by inhibiting the activity of α-glucosidase[7]. Thus, this suggests that walnut green husk polyphenols have hypoglycaemic potential and are strong candidates for hypoglycaemic active functional ingredients. However, the *in vivo* hypoglycemic effect of walnut green husk polyphenols has not been reported and its mechanism has not been elucidated.

Network pharmacology is a new discipline in drug research that breaks the limitations of the previous "single target, single drug" into a highly efficient "multi-target, multi-constituent", and is therefore often used to screen biologically active small molecules, identify metabolic pathways, and find the binding affinity between compounds and target proteins[8]. Traditional testing methods are time-consuming and labor-intensive, while using network pharmacology and bioinformatics can shorten the screening steps and result in more rapid screening of metabolic pathways. The use of network pharmacology to predict the functions of pharmacodynamic substances and their mechanism of action in complex Chinese medicine ingredients has become a trend[9].

In this study, we investigated the role of walnut green husk polyphenol extract (WGHP) in alleviating T2DM and examined the effects of WGHP on the structure and diversity of the gut microbiota in T2DM mice. Moreover, we analyzed the key signaling pathways of WGHP in the treatment of T2DM by network pharmacology, and verified the mechanism of action of WGHP in modulating the glucose-lowering of the hepatic IRS/PI3K/AKT pathway.

2. Materials and Methods

2.1 Preparation and analysis of WGHP

WGHP was extracted with reference to our previous methodology[10]. The purity of WGHP was 88% ± 0.01. The main chemical constituents of WGHP were identified by UPLC-MS/MS.

2.2 UPLC-MS/MS analysis

WGHP was identified by UPLC-MS/MS[11]. Liquid phase conditions: Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm), mobile phase A: ultrapure water (with 0.1% formic acid added), phase B is acetonitrile (with 0.1% formic acid added). The mobile phase gradient was divided into four stages, (1) 0.00-9.00 min: the proportion of B phase increased linearly from 5% to 95%; (2) 9.00-10 min: maintained at 95%; (3)

10.00-11.10 min: the proportion of B phase decreased to 5%; (4) 11.10-14.00 min: maintained at 5%. The flow rate was 0.35 mL/min; the column temperature was 40ºC; and the injection volume was 4 μL.

Mass spectrometry conditions: an electrospray ionization (ESI) source was used at 550ºC and scanned at 5500 V (positive ion mode) /-4500 V (negative ion mode); the ion source gas I (GSI), gas I (GSII), and curtain gas (CUR) were set to 50, 60, and 25 psi, respectively. The collision-induced ionization parameter was set to High. QQQ scans were performed using MRM mode with the collision gas (nitrogen) set to medium. DP and CE of individual MRM ion pairs were accomplished by further declustering potential (DP) and collision energy (CE) optimization. A specific set of MRM ion pairs was monitored in each period based on the metabolites eluting within each period.

2.3 Animals and treatment

Six-week-old male C57BL/6J mice, weighing (20 ± 2) g were purchased from Beijing Spefo Biotechnology Co. The animal protocols used in this study were reviewed and approved by the Institutional Animal Care Use Committee of Yunnan Agricultural University (YNAU-202107015). After one week of acclimatization, C57BL/6J mice were randomly assigned to a normal control group and the remaining mice were fed a high-fat diet (HFD) containing 20% protein, 20% carbohydrate, and 60% fat (Trophic Animal Feed High-Tech Co.). Following four weeks on the HFD, mice were intraperitoneally injected with 50 mg/kg body weight of streptozotocin (STZ) for three consecutive days, and fasting blood glucose (FBG) was measured after 72 hours. Mice with FBG values exceeding 11.1 mmol/L on two occasions were considered to have successfully developed T2DM and were divided into five groups: a model group (T2DM), a positive drug Metformin (Met) group at 100 mg/kg, and two walnut green husk polyphenol (WGHP) groups at 50 mg/kg and 100 mg/kg, respectively. Body weights were recorded, and FBG was measured weekly.

At the end of the five-week treatment period, mice were fasted for 12 hours, weighed, and euthanized. Samples of kidney, liver, pancreas, spleen, lung, heart, epididymal fat, mesenteric fat, and cecum contents were collected. Tissues were quickly weighed and sampled; some were fixed in 4% paraformaldehyde, while others were snap-frozen in liquid nitrogen and stored at -80°C for future analysis. Blood samples were allowed to clot at room temperature, centrifuged at 12,000 r/min for 10 minutes, and the serum was collected and stored at -80ºC.

2.4 Oral glucose tolerance test (OGTT)

An OGTT was conducted during the final week of the experiment. Mice were fasted for 12 hours and then gavaged with 2 g/kg of a D-glucose solution. Post-gavage, blood samples were collected from the tail vein at 0, 15, 30, 60, 90, and 120 minutes, and blood glucose levels were measured at each time point using a glucometer. The area under the curve (AUC) was calculated over the 0-120 minute period to assess oral glucose tolerance.

2.5 Biochemical analysis

Commercially available kits (Jiancheng, Nanjing, China) were used to detect serum levels of triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). The concentrations of interleukin-6 (IL-6), interleukin-1β (IL-1β), lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) were measured using ELISA kits (Meimian, Jiangsu, China). Malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH-Px) and total antioxidant capacity (TAOC) in liver were determined according to the instructions of the corresponding assay kits (Meimian, Jiangsu, China). All measurements were performed according to the manufacturer's instructions provided in the corresponding kits.

2.6 Histopathological analysis

Pancreatic samples were stored in 4% paraformaldehyde for subsequent use. The tissues were dehydrated using an ethanol gradient, followed by paraffin embedding. They were then sectioned into 5 μm slices, stained with hematoxylin & eosin (H&E), and examined for pathological changes under a microscope.

2.7 Sequencing of the 16S rRNA gene of gut microbiota

DNA extraction from the cecal contents was performed according to kit instructions, with 1% agarose gel electrophoresis used to assess DNA quality. The primer sequences for PCR amplification were an upstream primer 338F: ACTCCTACGGGGAGGCAGCAG and a downstream primer 806R: GGACTACHVGGGGTWTCTAAT. Amplification products were detected using 2% agarose gel electrophoresis, purified with the DNA Gel Recovery Purification Kit, and quantified using Qubit 4.0. Samples were mixed in the appropriate proportions based on the sequencing requirements. Libraries were constructed with the NEXTFLEX Rapid DNA-Seq Kit and sequenced on the Illumina Miseq PE300/NovaSeq PE250 platform. Raw sequences were quality-controlled using fastp (version 0.20.0) software and FLASH (version 1.2.7) for splicing. Noise reduction of optimized sequences post-QC splicing was performed using the DADA2 plugin in Qiime2, with default parameters. Amplicon Sequence Variants (ASVs) were taxonomically analyzed against the Silva 16S rRNA gene database (version 138) using the Naive Bayes (or Vsearch, or BLAST) classifier in Qiime2. Alpha diversity was analyzed using mothur software, and the Wilcoxon rank-sum test was employed to assess between-group differences in alpha diversity. The similarity of microbial community structures between samples was evaluated using PCoA scores based on the Bray-Curtis distance algorithm with R-3.3.1 (vegan) software. LEfSe analysis (Linear Discriminant Analysis Effect Size) with an LDA score threshold >3.8 and $P < 0.05$ was used to identify bacterial taxa that significantly differed in abundance from phylum to genus level between groups.

2.8 Analyzing the hypoglycaemic pathway of WGHP using network pharmacology

During the network pharmacology, 32 compounds were obtained from the analysis according to 2.2, and the top 10 compounds in terms of peak area were selected for analysis. Find the smiles numbers of these 10 compounds on the PubMed website (https://pubmed.ncbi.nlm.nih.gov/) and download the SDF structure files. According to the smiles number, select "Homo sapiens" in Swiss target prediction

(http://swisstargetprediction.ch/) to find the predicted target for each substance, upload the SDF structure file to PharmMapper website (https://lilab-ecust.cn/pharmmapper/index.html), select "Human Protein Targets Only" and download the Uniprot numbers for each substance, based on the uniprot number, find the Gene name on the UniProt website (https://www.uniprot.org/) (excluding those without a uniprot number) and remove duplicates to get the drug target.

In addition, using "Diabetes Mellitus, type 2" as the keyword, we found T2DM related targets in six disease databases: GeneCards (https://www.genecards.org/), TTD (https://db.idrblab.net/ttd/), OMIM (https://www.omim.org/), GEO (https://www.ncbi.nlm.nih.gov/geo/), and DisGeNET (https://www.disgenet.org/). Duplicates were aggregated and removed to obtain 1632 disease targets. A venn diagram was drawn based on the jvenn drawing tool (https://www.bioinformatics.com.cn/static/others/jvenn/example.html) to create a library of WGHP-T2DM intersection targets.

The intersecting targets were imported into the STRING database (https://string-db.org), and the interactions between the target proteins were investigated. Select "Multiple proteins", set "Organisms" to "Homo sapiens", and set the overall score to ≥0.4. Then delete the no interaction node to obtain the Protein-Protein interaction (PPI) relationship. Associated targets were exported through the STRING database and imported into the DAVID database (https://david.ncifcrf.gov/) for GO and KEGG enrichment analysis. Statistical significance of $P < 0.05$ was used as the threshold for screening potential signaling pathways, and the data were analyzed and visualized using the microbiotics database (www.bioinformatics.com.cn).

2.9 Western blot analysis

Mouse liver samples from the control, T2DM, MET, and 100 mg/kg groups were lysed using PIPA tissue lysis buffer (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) at a ratio of 1:10. After grinding for 10 minutes with a tissue mill homogenizer, the liver proteins were lysed for 30 minutes on ice and then centrifuged at 12,000 r/min for 10 minutes at 4°C to collect the supernatant. The supernatant was mixed with 25% loading buffer and boiled for 10 minutes at 95°C. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Merck Millipore, Massachusetts, USA). The PVDF membranes were incubated with primary antibodies against p-IRS-1 (CST 2381T, 1:1000), IRS-1 (CST #3407, 1:1000), PI3K (ab 191606, 1:1000), AKT (Proteintech 60203-2-Ig, 1:30000), p-AKT (Proteintech 66444-1-Ig, 1:5000), GLUT4 (Proteintech 66846-1-Ig, 1:3000), and β-Tubulin (Proteintech 66240-1-Ig, 1:3000) at 37°C for 12 hours, followed by incubation with HRP-conjugated secondary detection antibodies for 90 minutes at 25°C. Finally, the blots were detected using an ultrasensitive ECL chemiluminescence system (Biosharp, Beijing, China). Protein expression levels were quantified using Image J and expressed as a percentage of the respective control values.

2.10 Statistical analysis

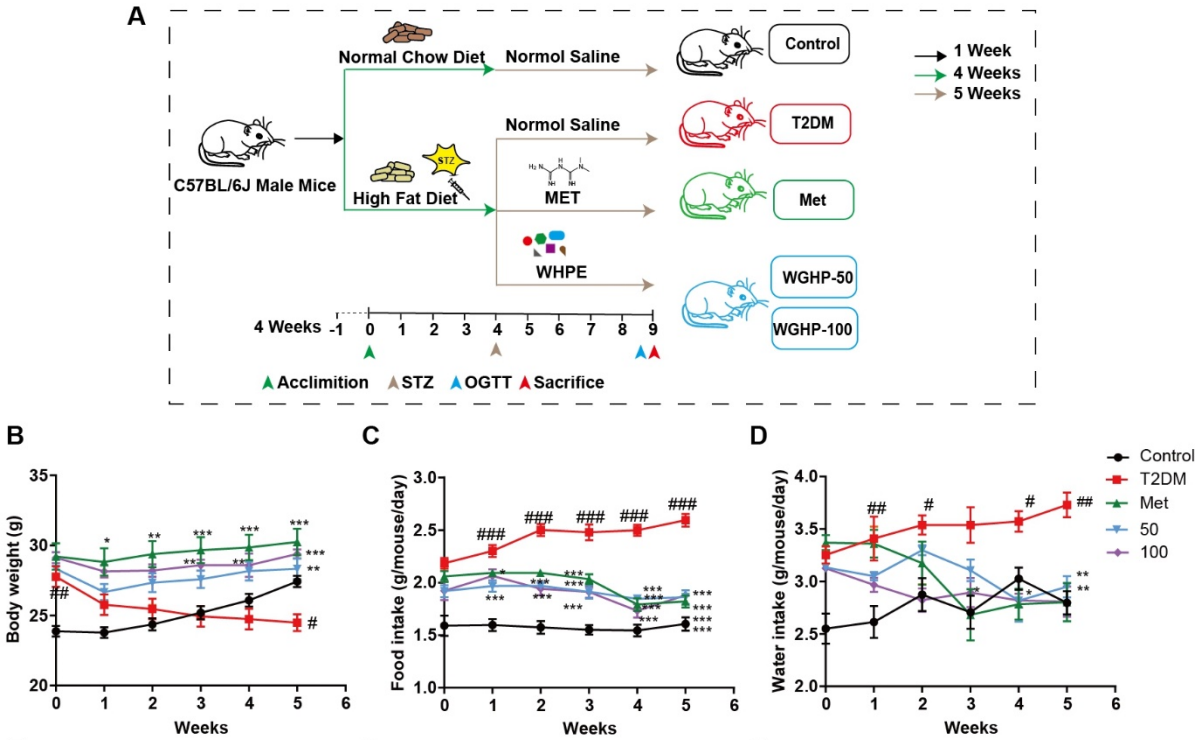
All experiments were repeated at least 3 times and data were expressed using mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) with

GraphPad Prism 8.0.2.263 software (Graph Pad Software, San Diego, USA). $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1 Effect of WGHP on body weight, food and water intake, and organ indexes in T2DM mice

To investigate the potential of WGHP in ameliorating diabetes mellitus, we established a mouse model of type 2 diabetes mellitus by HFD and streptozotocin, and then intervened with the Metformin, and different concentrations of WGHP for five weeks (Figure 1A). The development of T2DM is often accompanied by weight loss, and suppression of weight loss is considered to be a key piece of evidence for the success of treating T2DM[12]. We found that on the fifth week of gavage, the body weight of mice in the T2DM group decreased significantly compared to the control group, whereas the body weights of mice in the Met group, 50 mg/kg and 100 mg/kg WGHP groups increased significantly compared to the T2DM group (Figure 1B and E). In addition, T2DM mice showed a significant increase in food intake and water intake compared with the control group. On the contrary, Met and WGHP treatment significantly decreased the food intake (Figure 1C and F) and water intake (Figure 1D and G) compared with the T2DM mice. We also measured the weights of various tissues, and it was found that the liver, kidney and perinephric fat weight were significantly increased in the T2DM group of mice compared with the control group, and the Met and WGHP intervention treatments effectively blocked the HFD/STZ-induced increase in liver, kidney and perinephric fat weight (Figure 1H, I and J, Figure S1). Photographs of the livers further corroborate these findings(Figure S2). These results suggest that WGHP has the ability to inhibit weight loss, organ enlargement and abnormal increase in food and water intake in T2DM mice.



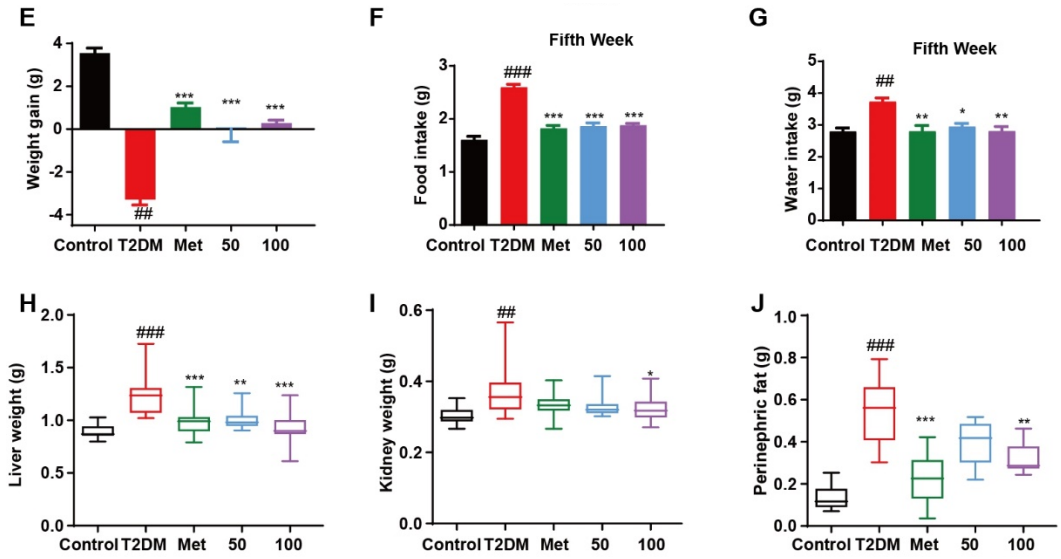
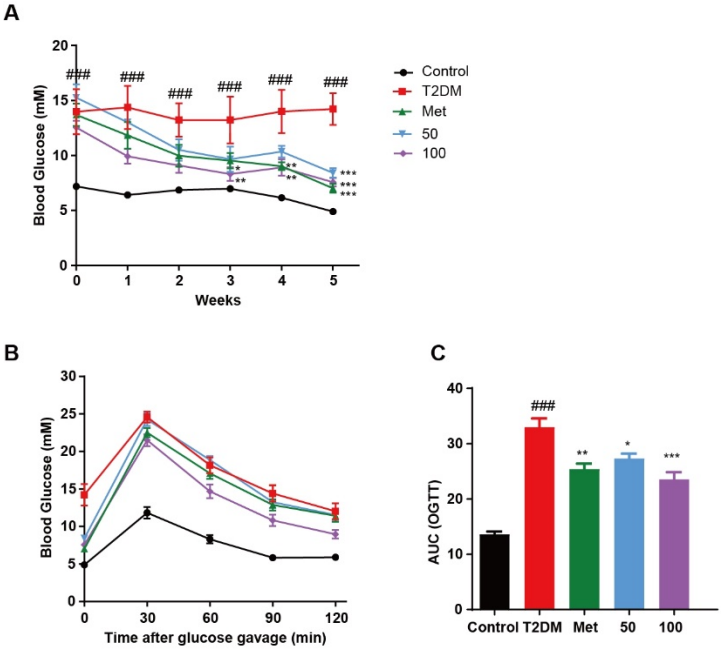


Figure 1 Effects of WGHP on body weight, diet, water intake and organ weights in mice. (A) Schematic diagram of the experimental design, (B) body weight, (C) body weight change from 1-5 weeks, (D) food intake from 1-5 weeks, (E) food intake in the fifth week, (F) water intake from 1-5 weeks, (G) water intake in the fifth week, (H) liver, (I) kidney and (J) perirenal fat weight. Data are presented as mean ± standard deviation of means (SEM, n = 12). #$P < 0.05$, ##$P < 0.01$, ###$P <$ 0.001 vs normal group,*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs T2DM group

3.2 Effect of WGHP on glycolipid metabolism in T2DM mice

To investigate the impact of WGHP on blood glucose levels in T2DM mice, fasting blood glucose (FBG) levels were monitored weekly using a glucose logger during the treatment period. As depicted in Figure 2A, the FBG levels in the T2DM group were significantly higher than those in the control group. However, after 5 weeks of treatment with Metformin (Met) and WGHP, the FBG levels in the T2DM group were significantly reduced compared to the untreated T2DM group. An oral glucose tolerance test (OGTT) was conducted, and the results are presented in Figure 2B and C. We observed that the area under the curve (AUC) was significantly increased in the T2DM group compared to the control group, but significantly decreased in Metformin- and WGHP-treated mice compared to the T2DM group. These findings indicate that WGHP significantly improves glucose tolerance in T2DM mice.



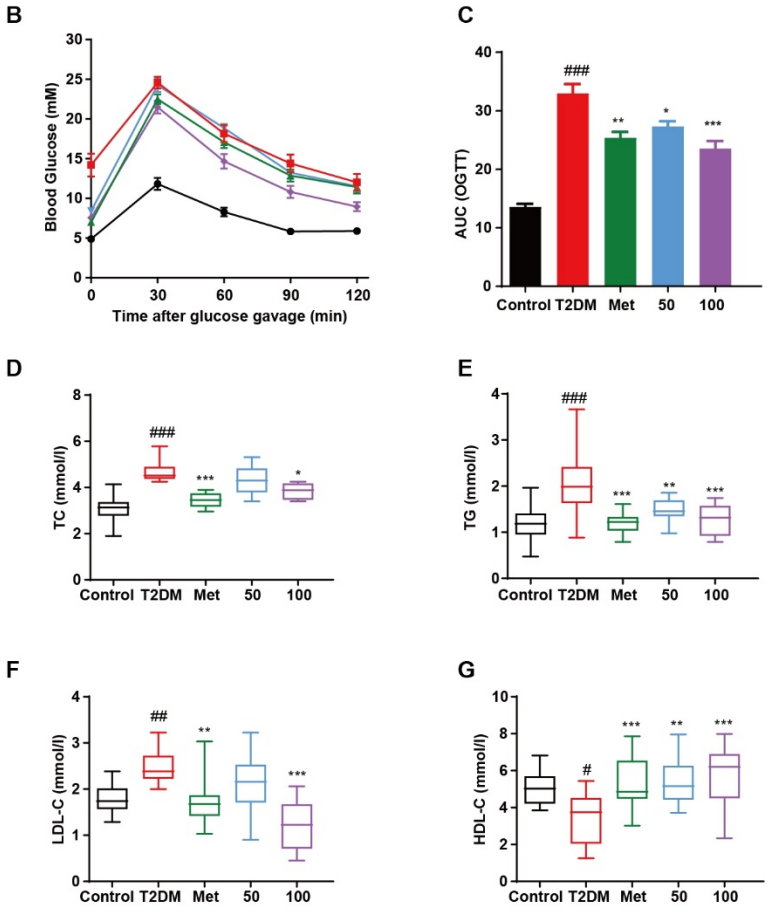


Figure 2 Effect of WGHP on glycolipid metabolism in T2DM mice. (A) FBG, (B) OGTT, (C) AUC and (D) TC, (E) TG, (F) LDL-C, (G) HDL-C in serum. Data are presented as mean ± SEM (n=12). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control group,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs.T2DM group

To assess the effects of WGHP on lipid metabolism in T2DM mice, we measured blood lipid levels. As shown in Figure 2D-F, serum levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) were significantly elevated in the T2DM group compared to the control group, while high-density lipoprotein cholesterol (HDL-C) levels were significantly lower than those in the control group. These alterations were significantly reversed in T2DM mice following Metformin and WGHP interventions. Collectively, these results demonstrate that WGHP effectively ameliorates the glycolipid metabolic disorders induced by T2DM.

3.3 Effect of WGHP on pancreatic histopathology, inflammatory response, liver function and oxidative stress in T2DM mice

Maintenance of normal pancreatic function is important in the prevention and treatment of T2DM [13]. To investigate the effect of WGHP on pancreatic histopathology in T2DM mice, we performed H&E staining of pancreatic tissue. Compared with the control group, the T2DM group had no obvious boundary between pancreatic islets and pancreatic alveolus cells, disturbed cell arrangement, islet atrophy, inflammatory cell infiltration, and increased cell vacuolization. However, compared with the T2DM group, the Met and WGHP groups showed clear and normal islet structure, clear boundaries between pancreatic alveolus and pancreatic islets, tightly arranged and regular cell arrangement, and decreased inflammatory cell infiltration and cell vacuolization (Figure 3A).

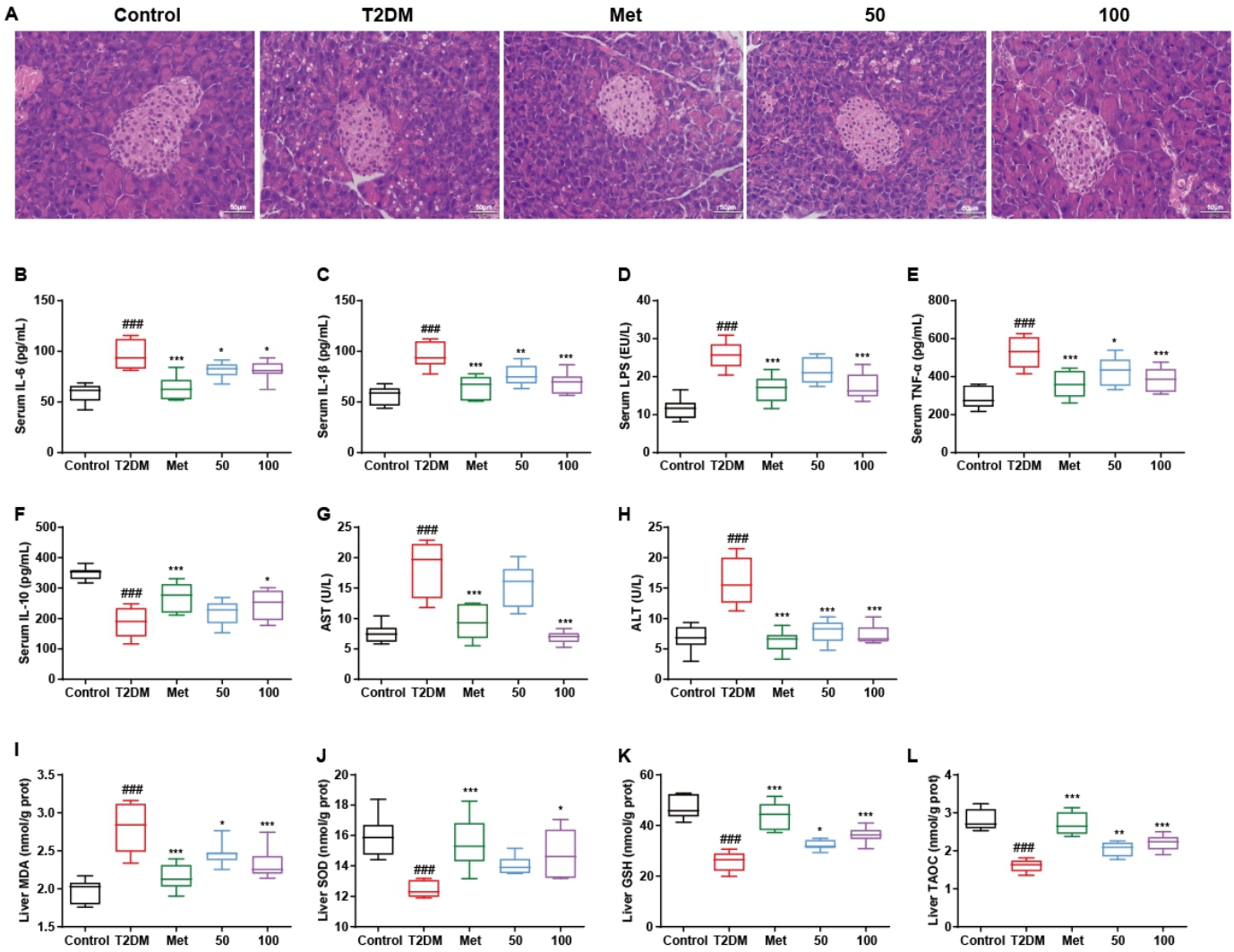


Figure 3 Effect of WGHP on pancreatic histopathology, inflammatory response, liver function and oxidative stress in T2DM mice. (A) H&E staining of the pancreas (400×magnification), the effect of WGHP on serum (B) IL-6, (C) IL-1β, (D) LPS, (E) TNF-α, (F) IL-10, (G) AST, (H) ALT, and liver (I) MDA, (J) SOD, (K) GSH, and (L) TAOC. Data are expressed as mean ± SEM (n=12). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs.T2DM group

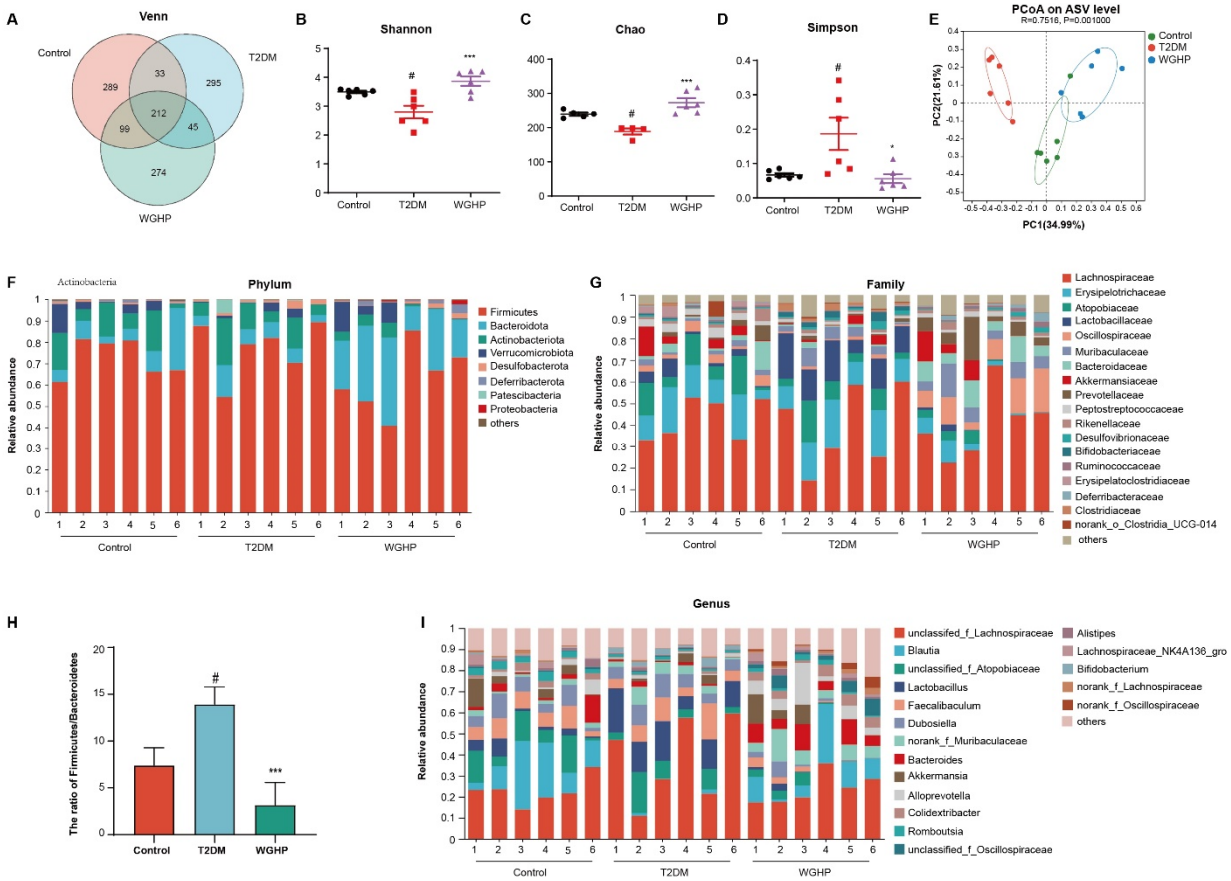
Since the pathogenesis of diabetes is accompanied by elevated levels of inflammatory factors [14], we examined serum inflammatory factor levels in mice. As shown in Figure 3B-F, serum levels of IL-6, IL-1β, TNF-α, and LPS were significantly elevated in the T2DM group of mice compared to the control group, while IL-10 levels were significantly lower. However, WHGP significantly reversed these symptoms in T2DM mice.

To investigate the effects of WGHP on liver function in T2DM mice, we examined serum ALT and AST levels. We found that AST and ALT were significantly increased in the T2DM groups of mice compared to the control group, while AST and ALT were significantly decreased in the Met and WGHP groups of mice compared to the T2DM group (Figure 3G and H). Since persistent hyperglycaemia induces chronic oxidative stress, we examined liver oxidative stress-related indices. As shown in Figure 3I-L, oxidative stress was increased in T2DM mice compared to controls, whereas Met and WGHP treatments reduced oxidative stress in T2DM mice, i.e., MDA levels were significantly decreased and SOD, GSH, and TAOC levels were

significantly increased. These results suggest that WGHP can ameliorate pancreatic injury, inflammatory response and hepatic oxidative stress in T2DM mice.

3.4 Effect of WGHP on the structure and diversity of gut microbiota in T2DM mice

There is a growing body of evidence suggesting that the pathogenesis of T2DM is closely related to gut microbiota imbalance[15]. To assess whether WGHP can modulate the structure and diversity of the gut microbiota in T2DM mice, we conducted a 16S rRNA analysis of the contents of the mouse cecum. The analysis of ASVs and the Venn diagram revealed the unique and shared species counts among the three sample groups. As depicted in Figure 4A, a total of 212 operational taxonomic units (OTUs) were identified across the control, T2DM, and WGHP groups, with 289, 295, and 274 OTUs unique to each group, respectively. Comparative analysis using the Chao and Shannon diversity indices revealed significant decreases in diversity and an increased Simpson index in the T2DM group compared to the control group. In contrast, supplementation with WGHP resulted in significantly increased Chao and Shannon indices and a decreased Simpson index compared to the T2DM group (Figure 4B-D). Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarities showed distinct clustering of the gut microbiota between the control and T2DM groups. After WGHP supplementation, the gut microbiota of the WGHP group showed a separation from the T2DM group and a partial overlap with the control group, indicating a microbiota profile that is more similar to the control (Figure 4E).



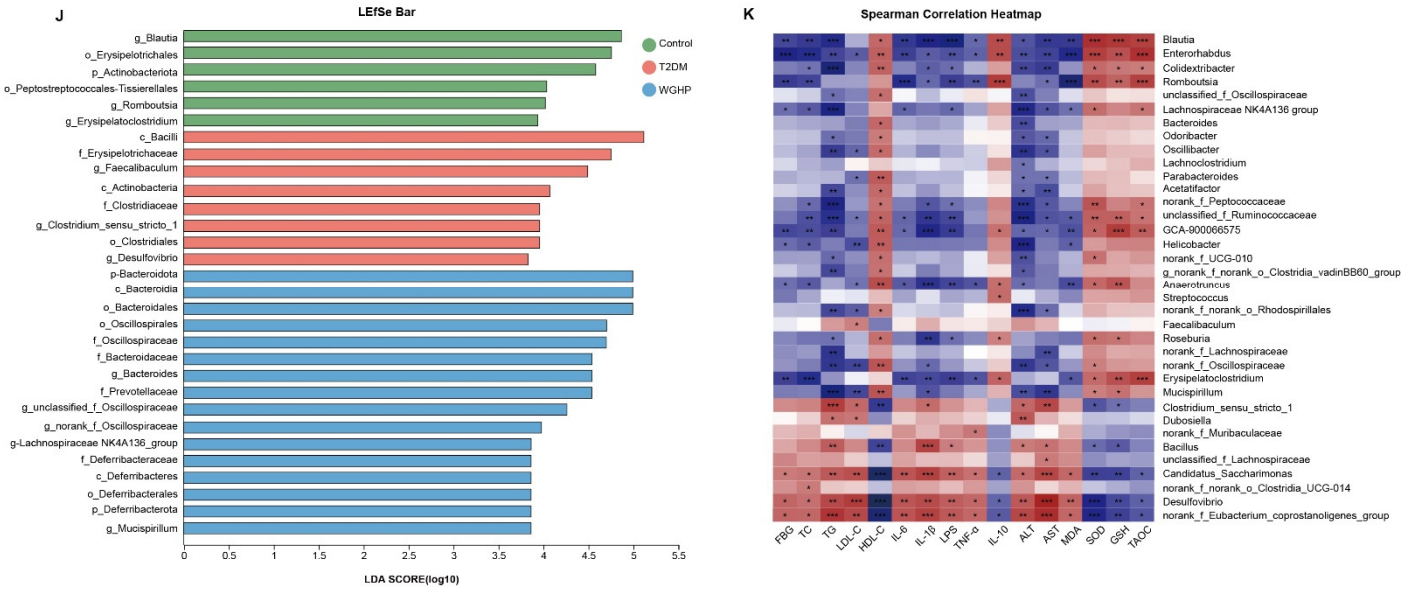


Figure 4 Effect of WGHP on gut microbes in T2DM mice. (A) Differences in gut microbial composition, the effect of WGHP (100mg/kg) on α-diversity (B) shannon index, (C) chao index, (D) simpson index and (E) principal coordinate analysis (PCoA), microbial composition at phylum, family, and genus level (F, G, I), (H) Firmicutes/Bacteroidetes (F/B), the and (J) LefSe analysis of gut microbes, and (K) Spearman correlation between gut microbiota and T2DM-related variables. Data are expressed as mean ± SEM (n = 6). $^{\#}P < 0.05$ vs. normal group,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs.T2DM group

To delve deeper into the impact of WGHP on the gut microbiota of T2DM mice, we conducted a comprehensive analysis of the microbial structure at the phylum, family, and genus levels within the mice's gut. Our analysis revealed that in the T2DM group, there was a notable increase in Firmicutes and a corresponding decrease in Bacteroidota, resulting in a higher Firmicutes/Bacteroidota (F/B) ratio when compared to the control group. Upon WGHP administration, we observed a reversal of these trends in T2DM mice, with a reduced abundance of Firmicutes and an increased abundance of Bacteroidota, alongside a significant reduction in the F/B ratio, as illustrated in Figure 4H and Figure S3 A and B. Furthermore, we noted a tendency for reduced Verrucomicrobia and Deferribacterota levels in the T2DM group; however, WGHP treatment notably elevated the levels of Deferribacterota in these mice (Figure S3C and D). Family-level analysis demonstrated that WGHP significantly enhanced the levels of Oscillospiraceae, Bacteroidaceae, Prevotellaceae, Ruminococcaceae, and Deferribacteraceae, while it decreased the levels of Erysipelotrichaceae, Lactobacillaceae, and Clostridiaceae in T2DM mice(Figure S4). At the genus level, WGHP significantly increased the level of *Bacteroides*, *Colidextribacter*, *g_unclassified_f_Oscillospiraceae*, and *g_norank_f_Lachnospiracea* in T2DM mice. Moreover, the levels of *Blautia*, *Akkermansia*, *Romboutsia*, *Alistipes*, and *Lachnospiraceae_NK4A136_group* also showed an improving trend following WGHP supplementation(Figure 4G and I, and Figure S5). These findings underscore the capacity of WGHP to modulate the gut microbiota composition in T2DM mice, highlighting its potential as a therapeutic agent for modulating gut microbial imbalances associated with T2DM.

To compare the dominant bacteria among the three groups, samples from each treatment group were subjected to linear discriminant analysis (LDA) using LEfSe analysis. The results shown in Figure 4J indicate that Firmicutes to *Erysipelatoclostridium*, Firmicutes to *Blautia*, Firmicutes to *Romboutsia* are the core group

of bacteria in the control group. Desulfobacterota to *Desulfovibrio*, Firmicutes to *Faecalibaculum*, and Firmicutes to *Clostridium_sensu_stricto_1* were the dominant organisms in the T2DM group. The WGHP group was mainly enriched in Bacteroidota to *Bacteroides*, Deferribacterota to Deferribacteraceae, Deferribacterota to *Mucispirillum*.

To investigate whether the ameliorative effect of WGHP on T2DM in mice is related to the gut microbiota, we performed Spearman correlation analysis between the gut microbiota and biochemical indices in mice. As shown in Fig. 4K, a total of 36 genera were correlated with at least one index. *Blautia*, Enterorhabdus, *Romboutsia*, *Erysipelatoclostridium*, *Enterorhabdus*, *Helicobacter*, *Anaerotruncus* show a significant negative correlation with pro-diabetic factors such as FBG, TC, TG, LDL-C, IL-6, IL-1β, LPS, TNF-α, ALT, AST, and MDA, and a significant positive correlation with anti-diabetic factors such as HDL-C, IL-10, SOD, GSH, and TAOC. In contrast, Clostridium_sensu_stricto_1, *Bacillus*, *Candidatus_Saccharimonas*, *Desulfovibrio*,*norank_f_Eubacterium_coprostanoligenes_group* showed significant positive correlation with FBG, TC, TG, LDL-C, IL-6, IL-Iβ, LPS, TNF-α, ALT, AST, and MDA, and significantly negatively correlated with HDL-C, IL-10, SOD, GSH, and TAOC. These results suggest that WGHP ameliorates gut microbiota disorders in T2DM mice, and the modulatory effect of WGHP on gut microbiota correlates with its amelioration of T2DM.

3.5 UPLC-MS/MS analysis of WGHP

To further elucidate the material composition of walnut green husk polyphenols (WGHP), we conducted a UPLC-MS/MS analysis of its components. The total ion chromatogram (XIC) results are presented in Figure S6. The mass spectrometry data for the main components of WGHP, including retention time (Rt), quasi-molecular ions ([M-H-]), and their characteristic ion fragments, are detailed in Table S1. The secondary mass spectrometry information is displayed in Figure S7. The ten substances analyzed included 6-O-Galloyl-β-D-glucose, 1-O-Feruloyl-β-D-glucose, Neochlorogenic acid, 6-O-Feruloyl-β-D-glucose, 1-Caffeoylquinic acid, Chlorogenic acid, Gallic Acid Ethyl Ester, p-Coumaric acid-4-O-glucoside, 1-O-Caffeoyl-β-D-glucose, and Phthalic anhydride. The structural formulas of these ten substances are presented in Figure S8.

3.6 Network pharmacological analysis of key components of WGHP

To elucidate the antidiabetic mechanisms of walnut green husk polyphenols (WGHP), we conducted a network pharmacological analysis. Utilizing the Swiss target prediction website and the PharmMapper database, we obtained 77 and 430 drug targets, respectively, with 491 unique drug targets after removing duplicates (Figure 5A). Furthermore, by leveraging GeneCards, TTD, OMIM, GEO, and DisGeNET databases, we aggregated and de-duplicated to obtain 1632 disease targets (Figure 5B).

The drug targets and T2DM targets were imported into the jvenn drawing tool, yielding 83 overlapping targets. These overlapping targets were then imported into the STRING database to establish a protein-protein interaction (PPI) network. The PPI network diagram was subsequently imported into Cytoscape network analysis software for analysis using the CytoNCA plugin. Key nodes were screened based on Betweenness,

Closeness, Degree, Eigenvector, and LAC, with all network values exceeding the median. After screening, 15 key targets were identified (Figure 5C, Table S2). The primary targets included AKT1, TNF, MMP9, EGFR, and PPARG. The results indicate that these genes are integral to the PPI network and are significant targets for WGHP in the treatment of T2DM.

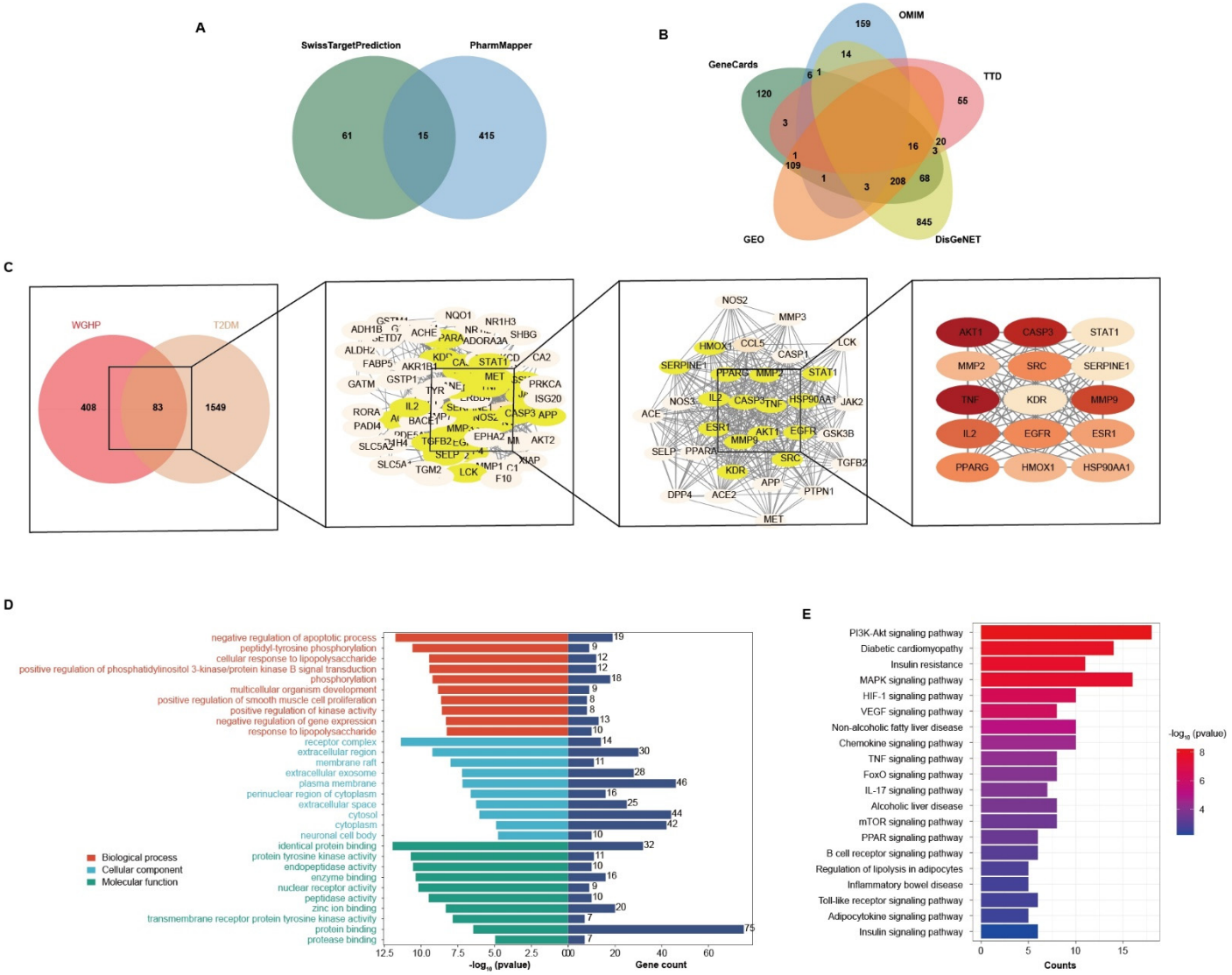


Figure 5 Network pharmacological analysis of WGHP in T2DM. (A) Veen diagrams of WGHP targets in SwissTarget and PharmMpper databases, (B) Veen diagrams of T2DM-related targets, and (C) screening process of key targets for WGHP action in T2DM. (D) GO enrichment analysis, and (D) KEGG enrichment analysis. The first 20 pathways with corresponding p-values are shown as scatter plots. The color scale represents the *p*-value and the size of the dots represents the number of genes in each pathway.

Additionally, we performed Gene Ontology (GO) enrichment analysis on the 83 core targets. The main biological processes involved were positive regulation of phosphatidylinositol 3-kinase/protein kinase B signal transduction, phosphorylation, and positive regulation of kinase activity, among others. The primary cellular components included receptor complexes, extracellular regions, membrane rafts, and extracellular exosomes. The main molecular functions were identical protein binding, protein tyrosine kinase activity, endopeptidase activity, and enzyme binding, etc. (Figure 5D).

KEGG pathway enrichment analysis was conducted on the 83 core targets using the DAVID database and microbiology platform. A total of 136 signaling pathways were obtained with a statistical significance of

$P < 0.05$, and 20 pathways closely related to glucose metabolism were selected. As shown in Figure 5E, the pathways related to T2DM by WGHP mainly included the PI3K-AKT signaling pathway, diabetic cardiomyopathy, insulin resistance, and the MAPK signaling pathway. Among them, the PI3K/AKT pathway was the most significantly enriched, which is crucial for the regulation of glucose metabolism and insulin resistance. Therefore, in subsequent studies, we will focus on the role of WGHP in regulating the hepatic PI3K/AKT signaling pathway.

3.7 Effect of WGHP on hepatic PI3K/AKT signaling pathway

It is known that the activation of insulin receptor kinases initiates the phosphorylation of insulin receptor subunits, such as insulin receptor substrate 1 (IRS-1). The activated IRS-1 further activates the PI3K/AKT pathway, which subsequently regulates glucose transport [16]. We observed that the protein expression levels of p-IRS1, PI3K, p-AKT, and GLUT4 were significantly lower in the T2DM group compared to the control group. Conversely, in the WGHP group, the protein expression levels of p-IRS1, PI3K, p-AKT, and GLUT4 were significantly higher compared to the T2DM group (Figure 6). The results indicate that WGHP enhances hepatic tissue glucose transport by increasing IRS1 phosphorylation, activating the PI3K/AKT pathway, and promoting GLUT4 protein expression.

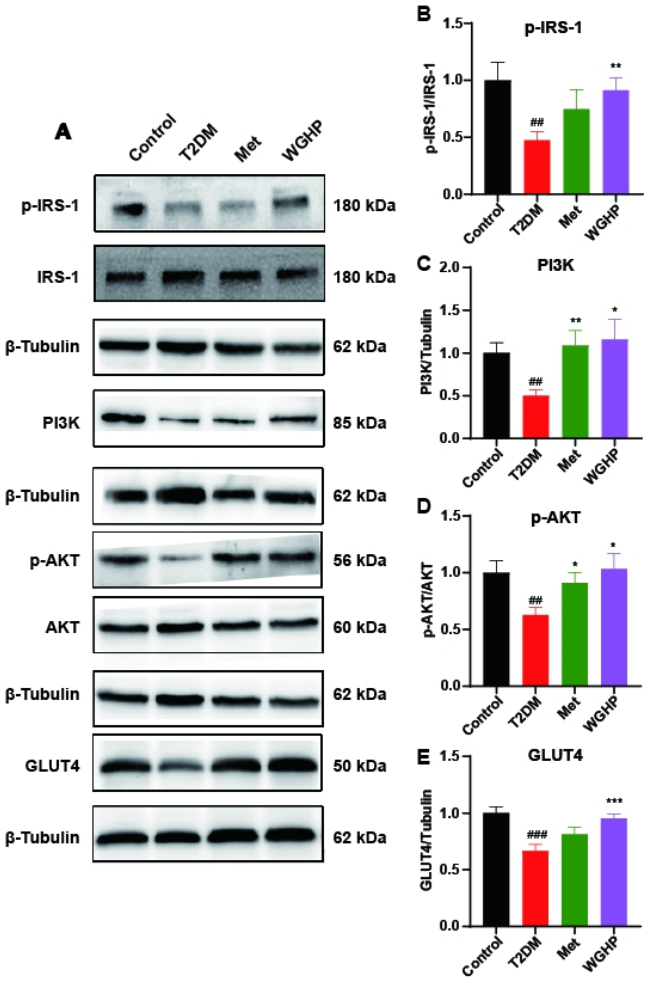


Figure 6 Effect of WGHP on PI3K/AKT signalling pathway in T2DM mice. (A) Representative western blot images of p-IRS-1, IRS-1, PI3K, p-AKT, AKT, GLUT4 and β-tubulin in control, T2DM, and WGHP group (100mg/kg) mice. Ratio of (B) p-IRS-1/ IRS-1, (C) PI3K/β-tubulin, p-AKT/AKT, and (E) GLUT4/β-tubulin. Data are expressed as mean ± standard deviation of means (n = 6). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control group,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs.T2DM group.

4. Discussion

Walnut green husk is a by-product of walnut processing, and rich in resources. Nevertheless the degree of exploitation is still very low. Walnut green husk is rich in bioactive substances, with a variety of physiological activities[17-21]. Recent studies have found that WGHP is beneficial in preventing obesity, non-alcoholic fatty liver disease, vascular endothelial dysfunction and colonic tissue damage[22], as well as controlling postprandial glucose levels by inhibiting α-glucosidase activity[10, 23]. However, the *in vivo* hypoglycaemic effects of walnut green husk polyphenols and their mechanism of action have not been reported. In this study, we demonstrated that WGHP has the ability to ameliorate the abnormalities of glucose and lipid metabolism, alleviate pancreatic injury, and attenuate inflammatory response and oxidative stress in T2DM mice. Then, we revealed the mechanism of action of WGHP in regulating the gut microbiota and activating the IRS-1/PI3K/AKT signaling pathway to regulate glucose metabolism in T2DM mice.

Studies have confirmed the anti-diabetic effects of polyphenolic substances found in fruits and vegetables[24]. In this study, we found that WGHP significantly ameliorated the 'three more and one less' symptoms and organ weight gain in T2DM mice induced by a high fat diet (HFD) and streptozotocin (STZ). This is consistent with the previous findings of Qi that Fu Brick Tea inhibited weight loss and abnormal increase in food and water intake in T2DM mice[25]. In addition, disorders of glycolipid metabolism triggered by a high fat diet (HFD) is the most important pathological features of T2DM[26]. Fortunately, our study demonstrated that supplementation with WGHP significantly ameliorated the abnormalities of glycolipid metabolism in T2DM. Our results are consistent with the findings of Li et al. that polyphenol-rich walnut meal extract modulates abnormal glycolipid metabolism in T2DM rats[27]. In addition, T2DM is associated with elevated levels of inflammation, oxidative stress and impaired liver function[28]. The release of pro-inflammatory factors activates oxidative stress in the tissues, exacerbating the disruption of glycolipid metabolism and creating a vicious circle[14]. Upon analysis, it was observed that WGHP (Whole Grain High Protein) intervention exerted significant effects on cytokine levels and oxidative stress markers in T2DM (Type 2 Diabetes Mellitus) mice. Specifically, WGHP led to a noteworthy decrease in pro-inflammatory cytokines IL-6, IL-1β, LPS, and TNF-α, accompanied by a marked increase in the anti-inflammatory cytokine IL-10. This modulation suggests a potential regulatory role of WGHP in immune response pathways associated with T2DM. Moreover, WGHP intervention demonstrated a mitigating effect on oxidative stress in T2DM mice, as evidenced by a substantial reduction in malondialdehyde (MDA) levels, indicative of lipid peroxidation, and an increase in superoxide dismutase (SOD), glutathione (GSH), and total antioxidant capacity (TAOC) levels. These findings imply a protective mechanism against oxidative damage induced by diabetes-related metabolic disturbances. Furthermore, WGHP intervention also significantly reduced the levels of liver enzymes glutamate transaminase (ALT) and glutamine transaminase (AST) in T2DM mice. This reduction suggests a potential improvement in liver function markers, reflecting the broader metabolic benefits of the intervention. This also confirms that WGHP can play an antidiabetic role by regulating glucose and lipid Metabolism, inflammatory factor levels and oxidative stress.

The gut microbiota is intricately linked to the development of diabetes, and its disruption can lead to a cascade of metabolic imbalances, including a reduction in short-chain fatty acids, altered metabolism of branched-chain amino acids, increased lipopolysaccharides, and endotoxin secretion, ultimately resulting in insulin resistance, chronic inflammation, and the progression to T2DM[29]. Our investigation into the effects of WGHP on the gut flora of T2DM mice revealed several key findings. Initially, we observed that WGHP increased the Shannon and Chao indices and decreased the Simpson index, which had been disrupted by T2DM. These changes indicate a restoration of microbial diversity and a shift towards a more balanced gut microbiota. Furthermore, the Firmicutes/Bacteroidetes (F/B) ratio, a critical marker of intestinal microbial balance, was significantly higher in the T2DM group but was effectively down-regulated by WGHP treatment, contributing to the amelioration of gut microbial dysbiosis[30]. This is in line with literature reports that polyphenols from germinated mung beans can modulate the Firmicutes and Bacteroides in T2DM mice, thereby improving the imbalance in the intestinal flora[31]. Importantly, WGHP supplementation was found to promote the growth of key beneficial bacteria such as *Blautia*, *Bacteroides*, *Akkermansia*, *Colidextribacter*, *Romboutsia*, *g__unclassified__f__Oscillospiraceae*, *Alistipes*, *g__Lachnospiraceae_NK4A136_group*. *Blautia*, in particular, has been negatively associated with T2DM and has been shown to inhibit lipid accumulation, body weight gain, and blood glucose elevation when supplemented orally in T2DM mice[32-33]. *Bacteroides* and *Akkermansia*, also negatively associated with T2DM, have been demonstrated to improve glucose intolerance and insulin resistance in diabetic mice, playing a beneficial role in glucose metabolism in both humans and experimental animals[34-36]. *Akkermansia muciniphila*, a promising gut microbiota, is known to stimulate intestinal wall integrity and may reduce inflammation, endoplasmic reticulum stress, adipogenesis, and gluconeogenesis, thereby improving T2DM[35]. Correlation analysis further validated these findings. In summary, WGHP treatment not only reverses the structural changes of the intestinal microbiota caused by T2DM but also reshapes the gut microbiota by increasing the diversity of intestinal microorganisms and the abundance of various beneficial bacteria.

The above results show that WGHP has positive ameliorative effects on T2DM and is a potent hypoglycaemic active compound. Therefore, in order to deeply reveal its mechanism of action, we predicted the pathway through which it exerts hypoglycaemic effects using network pharmacology. It provides a method for exploring and developing novel plant-based drugs that can be used to predict potential targets and multicomponent signaling pathways. Our study suggests that the PI3K/AKT signaling pathway plays a key role in the activity of WGHP on T2DM. Insulin receptor substrate (IRS) is a key protein in glucose-lipid metabolism, and phosphorylated IRS exposes binding sites for downstream protein binding. In signaling, insulin first binds to the α-subunit of the insulin receptor and promotes phosphorylation of its β-subunit tyrosine site, which in turn binds to IRS-1, and the phosphorylated IRS-1 interacts with the subunits of PI3K, and activated PI3K binds to lipotriphosphatidylinositol triphosphate (PIP3) through the second messenger, and lipotriphosphatidylinositol-dependent protein kinase (PDK)-1 and protein kinase C (PKC)

binding, which in turn activates AKT, and the activation of AKT promotes the translocation of glucose transporter vesicles to the cell membrane, accelerating the transport of the glucose transporter protein GLUT, and enhancing glucose uptake[37-38]. Reduced expression and dysfunction of glucose transporter protein 4 (GLUT4) are strongly associated with the development of insulin resistance. Thus, increasing GLUT4 expression is a promising strategy for the prevention and treatment of IR and T2DM[39].

Therefore, to verify the accuracy of network pharmacology, we investigated the effect of WGHP on the expression of relevant proteins in the liver of T2DM mice using western blotting. In our study, WGHP increased the level of phosphorylation of IRS1, which activates PI3K proteins, leading to increased phosphorylation of AKT and consequently increased GLUT4 expression. The experimental results verified the results of bioinformatics analysis, meanwhile, the experimental results were consistent with the results of previous studies. For example, the aqueous extract of Fu Brick tea alleviated T2DM-induced dyslipidaemia, insulin resistance and hepatic oxidative stress in mice and remodeled gut microbiota by activating the IRS1/PI3K/AKT pathway and modulating intestinal metabolites[25]. Choisy extracts can regulate fasting blood glucose, improve hepatic insulin sensitivity and promote hepatic glycogen storage, regulate lipid metabolism abnormality in T2DM mice, and reduce inflammation through activation of IRS1/PI3K/AKT pathway, exerting hypoglycaemic effect[3]. *Codonopsis Radix* and *Polygonati Rhizoma* can effectively improve lipid metabolism disorders, repair damaged liver tissues, repair damaged pancreatic tissues, and reduce insulin resistance in T2DM mice, and the mechanism of action is related to the activation of the IRS1/PI3K/AKT signaling pathway[40]. Taken together, the results of this study suggest that WGHP exerts a hypoglycaemic effect in T2DM mice by activating the IRS1/PI3K/AKT signaling pathway.

5. Conclusion

In this study, we used a combination of *in vivo* experiments, 16S rRNA and network pharmacology analysis to reveal the therapeutic efficacy and mechanism of action of WGHP on T2DM. In conclusion, WGHP ameliorated the abnormalities of glucose-lipid metabolism, mitigated pancreatic and hepatic injuries, and attenuated inflammatory responses and oxidative stress in T2DM mice. WGHP also improved the disorders of the gut microbiota in T2DM mice, and increased the populations of the beneficial bacteria, such as *Blautia*, *Bacteroides*, *Akkermansia*, and so on. In addition, WGHP alleviated glucose Metabolism disorders in T2DM mice by activating the IRS/PI3K/AKT signaling pathway. Thus, WGHP is a promising hypoglycaemic active substance, and these results provide a scientific basis for the development of novel antidiabetic products.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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References

[1] L. Chen, H. Teng, H. Cao, Chlorogenic acid and caffeic acid from Sonchus oleraceus Linn synergistically attenuate insulin resistance and modulate glucose uptake in HepG2 cells, Food and chemical toxicology. 127(2019) 182-187. https://doi.org/10.1016/j.fct.2019.03.038

[2] S. S. Schwartz, S. Epstein, B. E. Corkey, et al., The Time Is Right for a New Classification System for Diabetes: Rationale and Implications of the β-Cell-Centric Classification Schema, Diabetes Care. 39(2016) 179-86. https://doi.org/10.2337/dc15-1585

[3] D. X. Jin, J. F. He, X. et al., Zhang, Hypoglycemic effect of Hypericum attenuatum Choisy extracts on type 2 diabetes by regulating glucolipid metabolism and modulating gut microbiota, Journal of Functional Foods. 52(2019) 479-491. https://doi.org/10.1016/j.jff.2018.11.031

[4] J. M. Pappachan, C. J. Fernandez, E. C. Chacko, Diabesity and antidiabetic drugs, Molecular Aspects of Medicine. 66(2019) 3-12. https://doi.org/10.1016/j.mam.2018.10.004

[5] C. Ciniglia, C. Sansone, L. Panzella, et al., Effects of walnut husk washing waters and their phenolic constituents on horticultural species, Environmental Science and Pollution Research. 19(2012) 3299-3306. https://doi.org/10.1007/s11356-012-0847-7

[6] A. Jahanban-Esfahlan, A. Ostadrahimi, M. Tabibiazar, et al., A Comprehensive Review on the Chemical Constituents and Functional Uses of Walnut (*Juglans spp*.) Husk, International journal of molecular sciences. 20(2019) 3920. https://doi.org/10.3390/ijms20163920

[7] G. Wang, X. Yang, J. Wang, et al., Walnut green husk polysaccharides prevent obesity, chronic inflammatory responses, nonalcoholic fatty liver disease, and colonic tissue damage in high-fat diet-fed rats, Int J Biol Macromol. 182(2021) 879-898

[8] F. Noor, M. Asif, U. A. Ashfaq, et al., M. Machine learning for synergistic network pharmacology: a comprehensive overview, Briefings in Bioinformatics. 24(2023) 120. https://doi.org/10.1093/bib/bbad120

[9] L. Zhao, H. Zhang, N. Li, et al., Network pharmacology, a promising approach to reveal the pharmacology mechanism of Chinese medicine formula, Journal of Ethnopharmacology. 309(2023) 116306. https://doi.org/10.1016/j.jep.2023.116306

[10] X. Hu, J. Xie, Y. Y. Bai, et al., Extraction, *in vitro* hypoglycaemic activity and active ingredient analysis of polyphenols from walnut green husk, Journal of Functional Foods. 122(2024) 106508. https://doi.org/10.1016/j.jff.2024.106508

[11] J. Naibaho, A. Wojdyło, M. Korzeniowska, et al., Antioxidant activities and polyphenolic identification by UPLC-MS/MS of autoclaved brewers' spent grain, LWT. 163(2022) 113612. https://doi.org/10.1016/j.lwt.2022.113612

[12] M Abhishek, B. V. Somashekaraiah, S. M. Dharmesh, *In vivo* antidiabetic and antioxidant potential of *Psychotria dalzellii* in streptozotocin-induced diabetic rats, South African Journal of Botany. 121(2019), 494-499. https://doi.org/10.1016/j.sajb.2018.12.006

[13] Y. Liu, H. Huang, Z. Xu, et al., Fucoidan protects the pancreas and improves glucose metabolism through inhibiting inflammation and endoplasmic reticulum stress in T2DM rats, Food & Function. 13(2022) 2693-2709. https://doi.org/10.1039/d1fo04164a

[14]Y. Wang, J. Wang, S. Y. Tao, et al., Mitochondrial damage-associated molecular patterns: A new insight into metabolic inflammation in type 2 diabetes mellitus, Diabetes metabolism research and reviews. 40(2024) e3733. https://doi.org/10.1002/dmrr.3733

[15] X. Cao, X. Wang, Y. Ren, et al., *Lonicera caerulea* L. polyphenols improve short-chain fatty acid levels by reshaping the microbial structure of fermented feces *in vitro*, Frontiers in Microbiology. 14(2023) 12289700. https://doi.org/10.3389/fmicb.2023.1228700

[16] Z. Mo, M. Zhan, X. Yang, et al., Fermented dietary fiber from soy sauce residue exerts antidiabetic effects through regulating the PI3K/AKT signaling pathway and gut microbiota-SCFAs-GPRs axis in type 2 diabetic mellitus mice, International Journal of Biological Macromolecules. 270(2024) 132251. https://doi.org/10.1016/j.ijbiomac.2024.132251

[17] S. Fordos, N. Abid, M. Gulzar, et al., Recent development in the application of walnut processing by-products (walnut shell and walnut husk), Biomass Conversion and Biorefinery. 13(2023) 14389-14411. https://doi.org/10.1007/s13399-023-04778-6

[18] Y. Fulin, Y. Tiantian, Chemical Constituents and Antitumor Activities of Walnut Green Husk, Medicinal plant. 10(2019) 17–24.

[19] Z. W. Dong , Y. F. Yuan, Juglanin suppresses fibrosis and inflammation response caused by LPS in acute lung injury, Int J Mol Med. 41(2018) 3353–3365

[20] P. Abedi, M. Yaralizadeh, M. Fatahinia, et al., Comparison of the effects of *Juglans nigra* Green Husk and Clotrimazole on *Candida albicans* in rats, Jundishapur J Microbiol. 11(2017) e58151. https://doi.org/10.5812/jjm.58151

[21] X. Wang, D. Chen, Y. Li, et al., Alleviating effects of walnut green husk extract on disorders of lipid levels and gut bacteria flora in high fat diet-induced obesity rats, J Funct Foods. 52(2019) 576-586

[22] G. Wang, Q. Han, X. Yan, et al., Polyphenols-rich extracts from walnut green husk prevent non-alcoholic fatty liver disease, vascular endothelial dysfunction and colon tissue damage in rats induced by high-fat diet, Journal of Functional Foods. 87(2021) 104853. https://doi.org/10.1016/j.jff.2021.104853

[23] H. Shen, J. Wang, J. Ao, et al., Inhibitory kinetics and mechanism of active compounds in green walnut husk against α-glucosidase: Spectroscopy and molecular docking analyses, LWT. 172(2022) 114179. https://doi.org/10.1016/j.lwt.2022.114179

[24] R. Naz, F. Saqib, S. Awadallah, et al., Food Polyphenols and Type II Diabetes Mellitus: Pharmacology and Mechanisms, Molecules. 28(2023) 3996. https://doi.org/10.3390/molecules28103996

[25] B. Qi, D. Ren, T. Li, et al., Fu Brick Tea Manages HFD/STZ-Induced Type 2 Diabetes by Regulating the Gut Microbiota and Activating the IRS1/PI3K/Akt Signaling Pathway, Journal of Agricultural and Food Chemistry. 70(2022) 8274-8287. https://doi.org/10.1021/acs.jafc.2c02400

[26] D. Wang, L. Yang,Y. Liu, Targeting AMPK Signaling in the Liver: Implications for Obesity and Type 2 Diabetes Mellitus, Current Drug Targets. 23(2022) 1057-1071. https://doi.org/10.2174/1389450123666220429082702

[27] Y. Li, D. Chen, C. Xu, et al., Glycolipid metabolism and liver transcriptomic analysis of the therapeutic effects of pressed degreased walnut meal extracts on type 2 diabetes mellitus rats, Food & Function. 11(2020) 5538-5552. https://doi.org/10.1039/d0fo00670j

[28] O. O. Oguntibeju, Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links, Int J Physiol Pathophysiol Pharmacol. 11(2019) 45-63.

[29] M. Su, T. Tang, W. Tang, et al., Astragalus improves intestinal barrier function and immunity by acting on intestinal microbiota to treat T2DM: a research review, Frontiers in Immunology. 14(2023) 124834. https://doi.org/10.3389/fimmu.2023.1243834

[30] A. Vaiserman, M Romanenko, L. Piven, et al., Differences in the gut Firmicutes to Bacteroidetes ratio across age groups in healthy Ukrainian population, BMC Microbiology. 20(2020) 221. https://doi.org/10.1186/s12866-020-01903-7

[31] X. Shen, X. Jiang, L. Qian, et al., Polyphenol Extracts From Germinated Mung Beans Can Improve Type 2 Diabetes in Mice by Regulating Intestinal Microflora and Inhibiting Inflammation, Frontiers in Nutrition. 9(2022) 846409. https://doi.org/10.3389/fnut.2022.846409

[32] K. Hosomi, M. Saito, J. Park, et al., Oral administration of *Blautia wexlerae* ameliorates obesity and type 2 diabetes via metabolic remodeling of the gut microbiota, Nature Communications. 13(2022) 4477. https://doi.org/10.1038/s41467-022-32015-7

[33] Q. Guo, Z. Gao, L. H. et al., Multiomics Analyses With Stool-Type Stratification in Patient Cohorts and Blautia Identification as a Potential Bacterial Modulator in Type 2 Diabetes Mellitus, Diabetes. 73(2024) 511-527. https://doi.org/10.2337/db23-0447

[34] M. Gurung, Z. Li, H. You, et al., Role of gut microbiota in type 2 diabetes pathophysiology, EBioMedicine. 51(2020) 102590. https://doi.org/ 10.1016/j.ebiom.2019.11.051

[35] P. G. Cano, A. Santacruz, Á. Moya, et al., *Bacteroides uniformis* CECT 7771 Ameliorates Metabolic and Immunological Dysfunction in Mice with High-Fat-Diet Induced Obesity, PLoS ONE. 7(2012) e41079. https://doi.org/10.1371/journal.pone.0041079

[36] M. I. D. Sanjiwani, I. P. H. Aryadi, I. M. S. Semadi, Review of Literature on *Akkermansia muciniphila* and its Possible Role in the Etiopathogenesis and Therapy of Type 2 Diabetes Mellitus, Journal of the ASEAN Federation of Endocrine Societies. 37(2022) 69-74. https://doi.org/ 10.15605/jafes.037.01.13

[37] M. C. Petersen, D. F. Vatner, G. I. Shulman, Regulation of hepatic glucose metabolism in health and disease, Nature Reviews Endocrinology. 13(2017) 572-587. https://doi.org/10.1038/nrendo.2017.80

[38] E. Caro-Gómez, J. A Sierra, J. A Escobar, et al., Green Coffee Extract Improves Cardiometabolic Parameters and Modulates Gut Microbiota in High-Fat-Diet-Fed ApoE(-/-) Mice, Nutrients. 11(2019) 497. https://doi.org/10.3390/nu11030497

[39] X. Yao, L. Liu, W. Shao, et al., Tectorigenin targets PKACα to promote GLUT4 expression in skeletal muscle and improve insulin resistance *in vitro* and *in vivo*, International Journal of Biological Sciences. 19(2023) 1579-1596. https://doi.org/10.7150/ijbs.80125

[40] Y. P. Mao, Y. M. Song, S. W. Pan, et al., Zhang, Effect of Codonopsis Radix and Polygonati Rhizoma on the regulation of the IRS1/PI3K/AKT signaling pathway in type 2 diabetic mice, Frontiers in Endocrinology. 13(2022) 1068555. https://doi.org/10.3389/fendo.2022.1068555