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Urolithin A improves insulin resistance by targeting TNF- α and regulating the intestinal microbiota

Yinghao Wang^{1,3†}, Chunfeng Li^{1†}, Wenyuan Peng¹, Jingning Yu¹, Jun Sheng^{1*}, Xuanjun Wang^{1,4*}, Xiaoyun Wu^{1,2*}

¹Key Laboratory of Puer Tea Science, Ministry of Education, Yunnan Agricultural University, Kunming, China

²Department of Science, Yunnan Agricultural University, Kunming, China

³Dabie Mountain Laboratory, College of Tea and Food Science, Xinyang Normal University, Xinyang, China

⁴College of Resources, Environment, and Chemistry, Chuxiong Normal University, Chuxiong, China.

ABSTRACT: Urolithin A (UA), an intestinal metabolite of ellagic acid, exhibits significant anti-inflammatory activity. Concurrently, Tumor necrosis factor α (TNF- α) serves as a pivotal inflammatory factor, and induces insulin resistance. This study aims to investigate the ameliorative effect of UA on TNF- α -induced insulin resistance and elucidate its underlying molecular mechanism. Methods and results: Through Western Blot (WB), glucose uptake, and immunocytochemistry (ICC) experiments, it was observed that UA significantly alleviated TNF- α -induced insulin resistance in C2C12 cells. Surface plasmon resonance (SPR) and molecular docking experiments validated the binding of UA to TNF- α with an affinity of 9.626×10^{-5} M, which demonstrated UA could directly bind to TNF- α and inhibiting its downstream signaling. These results suggested a potential monoclonal antibody-like mechanism of UA to inhibit TNF- α -induced insulin resistance. Furthermore, we also found the the substantial inhibitory effect of UA on LPS-induced pro-inflammatory polarization in RAW264.7 cells using WB and ICC experiments. In type 2 diabetes mellitus (T2DM) mouse models, OGTT, ITT, Elisa, WB, and RT-PCR experiments revealed that UA could effectively reduce blood glucose levels and serum concentrations of TNF- α and insulin while enhancing insulin signaling pathway transmission in muscle tissues and upregulating the expression of relevant genes for glycogen synthesis. Additionally, analysis of the intestinal microbiota showed that UA intervention markedly reshaped the composition and structure of the microbial community, reduced the elevated Firmicutes/Bacteroidetes ratio and increased the relative abundance of beneficial genera such as *Akkermansia*, *Dubosiella*, and *Kurthia*, as well as the key species *Akkermansia muciniphila*, thereby ameliorating HFD/STZ-induced dysbiosis and promoting the functional homeostasis of the intestinal ecosystem in T2DM. Conclusion: UA could directly bind to TNF- α and inhibit its downstream signaling, suggesting a potential monoclonal antibody-like mechanism. UA also exerted inhibitory effects on TNF- α -induced insulin resistance by specifically binding to TNF- α in C2C12 myotubes. We also found that UA could significantly improve insulin resistance, enhanced insulin signaling pathway transmission in muscle tissues, and improved intestinal microbiota dysbiosis.

Key words: Urolithin A, TNF- α , insulin resistance, T2DM, insulin sensitivity

[†] These authors contributed equally to this work.

***Corresponding author**

Xiaoyun Wu, wuxiaoyun79@163.com

Xuanjun Wang, wangxuanjun@gmail.com

Jun Sheng, shengj@ynau.edu.cn

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1. Introduction

Type 2 diabetes mellitus (T2DM) is the most prevalent form of diabetes mellitus, accounting for approximately 90% to 95% of all diabetic patients ^[1]. It is projected that by 2030, the number of individuals affected by T2DM will reach a staggering figure of 439 million ^[2]. Throughout its progression, T2DM presents with various short-term and long-term complications, including hypertension, dyslipidemia, coronary artery disease, stroke, retinopathy, nephropathy, neuropathy, and cancer ^[3]. The development of T2DM was regulated by multiple factors; however, insulin resistance serves as the primary cause ^[4, 5]. Research has indicated that obesity and inflammation played significant roles in promoting insulin resistance, which ultimately leads to T2DM ^[6]. Therefore, effective managements of obesity and inflammation can significantly suppress insulin resistance and subsequently improve T2DM.

Insulin resistance (IR), a pathological state characterized by reduced responsiveness of insulin target organs and elevated levels of serum insulin, serves as the underlying etiology for various metabolic disorders, including non-alcoholic fatty liver disease (NAFLD), atherosclerosis, and type 2 diabetes ^[5]. The development of IR can be attributed to factors such as obesity, inflammation, and pancreatic beta-cell atrophy. In recent years, considerable attention has been devoted to investigating inflammation-induced insulin resistance. Previous studies has revealed that pro-inflammatory cytokines, like TNF- α and IL-1 β , could activate JNK and IKK β /NF- κ B via classical receptor-mediated mechanisms, thereby impairing insulin signaling pathway transmission ^[7, 8]. Furthermore, research has demonstrated that metformin regulated IL-6-induced hepatic insulin resistance via activation of AMP-activated protein kinase (AMPK) ^[9]. Consequently, anti-inflammatory interventions have shown significant efficacy in ameliorating insulin resistance.

Tumor necrosis factor α (TNF- α) is a potent pro-inflammatory cytokine that exerts regulatory effects on various cell types and modulates the immune system. Additionally, it plays a pivotal role in promoting inflammatory reactions ^[10]. Apart from its immunomodulatory functions, TNF- α also influences insulin resistance regulation within the body ^[11]. Feingold et al. demonstrated that TNF- α induced impaired glucose tolerance in rodents ^[12]. Elevated levels of TNF- α expression have been observed in adipose tissues of obese individuals, and improvements of insulin sensitivity and glucose intolerance have been noted following neutralization ^[13, 14]. Moreover, a significant reduction in blood glucose levels was observed in patients with rheumatoid arthritis treated with anti-TNF- α drug infliximab ^[15]. As a monoclonal antibody, infliximab directly binds to TNF- α , thereby inhibiting the binding of TNF- α to its receptor and blocking the signaling pathway associated with TNF- α . Therefore, blocking TNF- α signaling pathway might represent an effective strategy for ameliorating inflammation-induced insulin resistance. However, biological TNF- α inhibitors (e.g., monoclonal antibodies) are costly and may pose immunosuppressive risks. Therefore, it is substantial interest in the identification of natural compounds with the capacity to safely modulate TNF- α activity. .

Urolithin A (UA), a metabolite of ellagic acid *in vivo*, exhibits diverse biological activities, including anti-inflammatory, antioxidant, and unique autophagy activation properties. Yaw Duah Boakye et al. discovered that UA exerted anti-inflammatory effects by inhibiting macrophage polarization ^[16]. Additionally,

UA can inhibit lipopolysaccharide-induced activation of NADPH oxidase (NOX) and suppress reactive oxygen species production ^[17]. Due to its multifaceted bioactivities, UA has demonstrated significant improvements in various metabolic diseases such as obesity, type 2 diabetes mellitus (T2DM), and non-alcoholic fatty liver disease (NAFLD) ^[18]. Studies have revealed that UA enhanced thermogenesis in brown adipose tissue and induced the browning of white adipose tissue by increasing triiodothyronine (T3) levels, thereby promoting energy expenditure and ameliorating obesity ^[19]. Moreover, numerous studies have shown the remarkable efficacy of UA in improving T2DM. It enhances mitochondrial function and biogenesis to improve insulin sensitivity ^[20], and prevents pancreatic β -cell apoptosis through autophagy ^[21]. However, despite these promising effects, the molecular mechanisms by which UA exerts its anti-inflammatory and insulin-sensitizing actions, particularly whether it involves direct binding to pivotal pro-inflammation cytokines like TNF- α , remain elusive. Therefore, based on its established anti-inflammatory activity and the central role of TNF- α in insulin resistance, we hypothesized that UA might function as a natural, direct inhibitor of TNF- α . This study specifically aims to test this hypothesis and investigate whether the direct targeting of TNF- α constitutes a primary mechanism through which UA ameliorates insulin resistance.

The purpose of this study is to investigate the direct binding between UA and TNF- α by combining surface plasmon resonance (SPR) and molecular docking, , and to validate the functional consequences of this interaction on insulin signaling and glucose metabolism using both *in vitro* and *in vivo* experiments. Additionally, we explore other potential mechanisms, including the modulation of gut microbiota. Notably, UA is not a direct dietary component but a gut microbiota-derived metabolite of ellagic acid, which is abundant in various foods such as pomegranates, berries, and nuts. Investigating the metabolic benefits of UA thus falls within the scope of food science and nutritional pharmacology, highlighting the crucial role of food-derived components and their microbial transformations in host health. Our work proposes, for the first time, that UA may act as a small-molecule direct inhibitor of TNF- α , which offers a novel perspective on its mechanism of action against T2DM.

2. Results

2.1 The structure of UA and its cytotoxicity experiment on cells

Using the MTT assay, we employed various concentrations of UA to assess its cytotoxicity on C2C12 cells. Following a 24-hour treatment with different concentrations of UA (ranging from 1.25 μ M to 80 μ M), we observed that although there was a significant decrease in cell viability at a concentration of 20 μ M compared to 0 μ M group (Figure 1B), the cell survival rate remained above 80%. Therefore, our subsequent experiments utilized a maximum dose of UA up to 20 μ M, ensuring non-induction of cellular toxicity for further investigations.

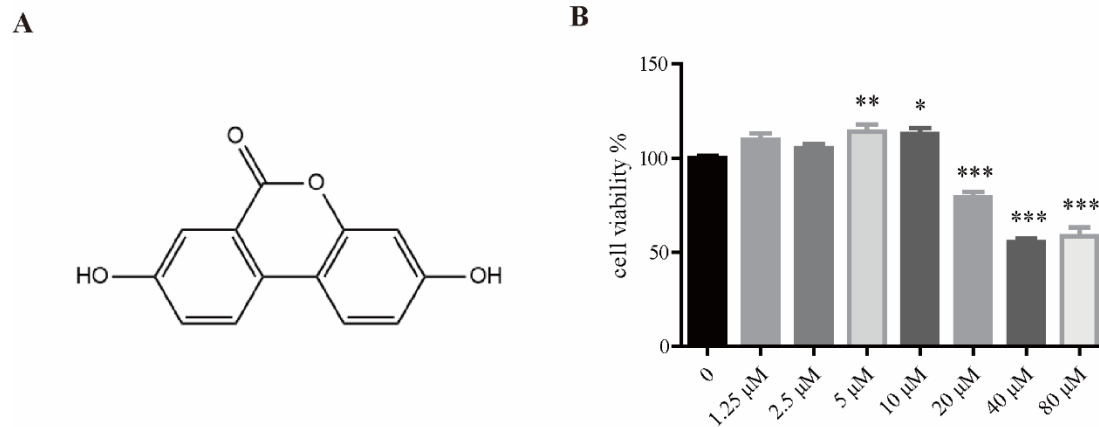
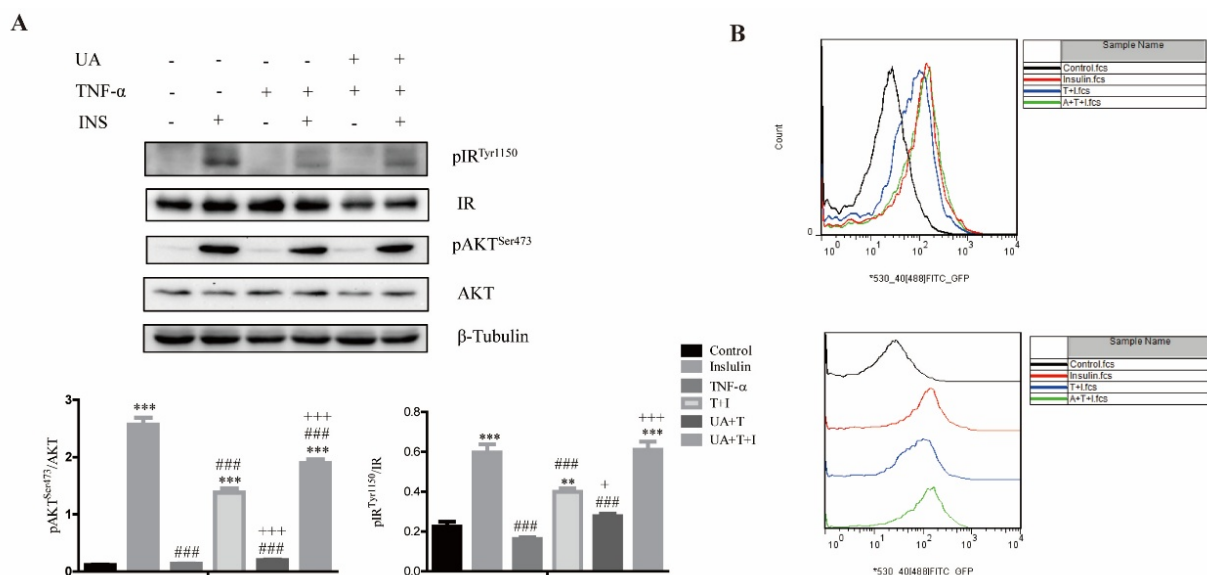


Figure 1. Chemical structure of Urolithin A and its cytotoxicity in C2C12 cells. (A) The chemical structure of Urolithin A; (B) the impact of various concentrations of UA on C2C12 cell toxicity after 24 hours, as assessed using the MTT assay. The data are expressed as mean $\pm$ SEM (n = 6). Compared to the 0 μ M group, statistical significance is denoted by * P < 0.05, ** P < 0.01, and *** P < 0.001.

2.2 The impact of UA on TNF- α -induced insulin resistance

Previous studies have demonstrated that TNF- α induces insulin resistance by inhibiting the insulin signaling pathway and suppressing the expression of Glut4, a key protein in glucose uptake [22, 23]. TNF- α has also been shown to impair the translocation of Glut4 to the cell membrane [24]. In this study, C2C12 cells were treated with UA (20 μ M) and TNF- α (20 ng/ml) for 24 hours. Insulin signaling was then stimulated with 100 nM insulin to examine the effect of UA on TNF- α -induced insulin resistance. The results revealed that TNF- α exposure markedly suppressed the expression of pIR^{Tyr1150} and pAKT^{Ser473}, key proteins in the insulin signaling pathway, as well as reduced cellular glucose uptake in insulin-stimulated C2C12 cells. However, co-treatment with UA effectively reversed these detrimental effects caused by TNF- α (Figure 2 A, B).

Confocal microscopy revealed that UA treatment significantly restored Glut4 translocation, which was reduced by TNF- α (Figure 2 C). Furthermore, TNF- α treatment markedly inhibited Glut4 expression in C2C12 cells; however, UA co-treatment remarkably increased its expression (Figure 2 D, E). These findings indicated that UA significantly ameliorated both TNF- α -induced insulin resistance and improved impaired glucose uptake capacity.



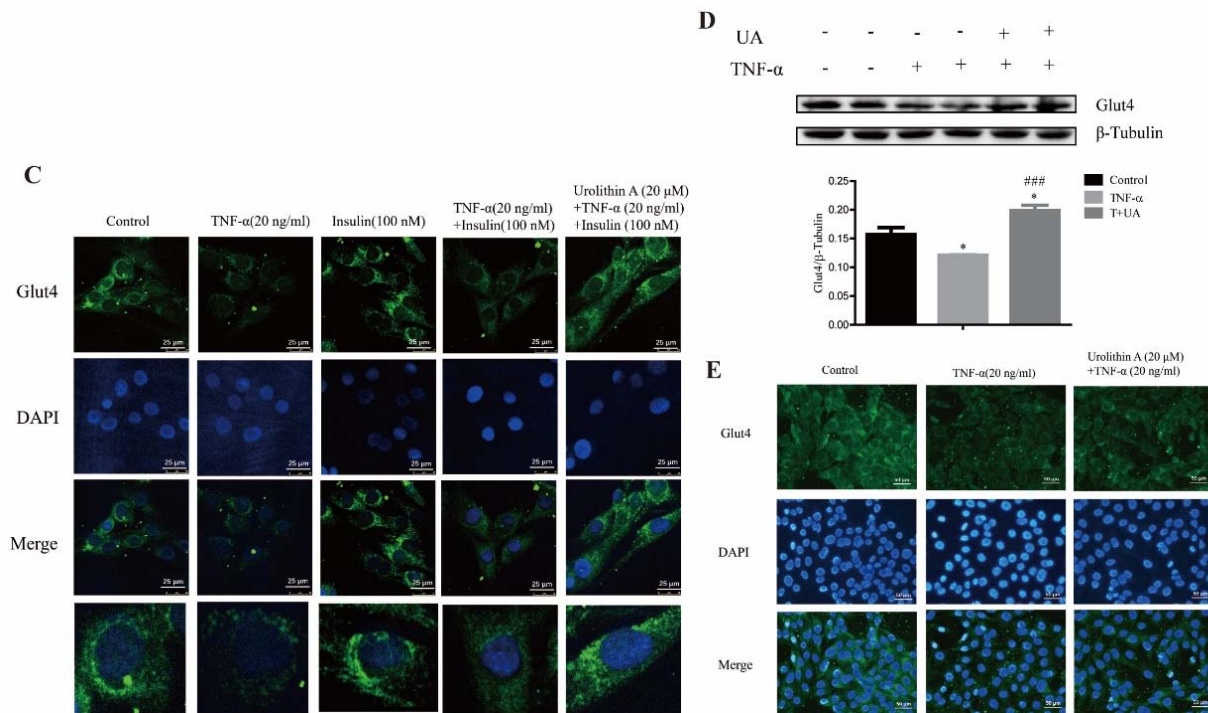


Figure 2. UA ameliorated TNF- α -induced impairments in insulin signaling and glucose uptake in C2C12 cells. (A) Western blotting technique was employed to analyze the alterations in expression levels of pIR^{Tyr1150}, IR, pAKT^{Ser473}, and AKT in C2C12 cells under different treatment conditions; (B) Flow cytometry was utilized to evaluate the impact on 2-NBDG uptake capacity following drug treatments; (C) Immunofluorescence experiments were conducted to visualize Glut4 localization, and confocal microscopy was used for examining changes in Glut4 protein expression patterns. Glut4 was labeled with green fluorescence while the cell nucleus was stained blue; (D) Western blotting technique was used to detect variations in Glut4 expression levels in C2C12 cells under different drug treatments; (E) Immunofluorescence experiments were performed to stain Glut4, and fluorescence inverted microscopy was employed for observing Glut4 expression. Glut4 is indicated by green labeling while the cell nucleus is marked blue. The values are represented as means $\pm$ SEM (n = 3). In Figure 2A, compared to the Control group, * P < 0.05, ** P < 0.01, *** P < 0.001; compared to the Insulin group, # P < 0.05, ## P < 0.01, ### P < 0.001; compared to the T+I group, + P < 0.05, ++ P < 0.01, +++ P < 0.001. In Figure 2A, compared to the Control group, * P < 0.05; compared to the TNF- α group, #### P < 0.001.

2.3 The binding between UA and TNF- α and its impact on the signaling pathway of TNF- α

TNF- α exerts its effects by binding to TNFR1. Infliximab and Adalimumab, monoclonal antibodies against TNF- α , bind to soluble TNF- α and neutralize it, thereby inhibiting the interaction between TNF- α and its receptor [25]. Research has shown that inhibiting TNF- α signaling is an effective strategy to suppress TNF- α -induced insulin resistance [11], and studies have also shown that Infliximab significantly improved blood glucose levels in the treatment of rheumatoid arthritis [26]. Therefore, we explored whether UA could function as a monoclonal antibody against TNF- α to block its effects. Utilizing SPR experiments, it was determined that UA exhibited an affinity of 9.626×10^{-5} M for TNF- α (Figure 3A). Molecular docking analysis revealed that UA bound to the "pocket" region of the TNF- α trimer (Figure 3B). AutoDock testing demonstrated a binding energy of -8.1 kcal/mol between UA and the TNF- α trimer structure (Figures 3C, D), while 2D display illustrated that hydrogen bonding and hydrophobic interactions primarily mediated the interaction between UA and the TNF- α trimer (Figure 3E). To further validate the impact of UA on the TNF- α signaling pathway and its effects, co-treatment with UA and TNF- α was performed on 3T3-L1 cells for a duration of 20 minutes. The results indicated that co-treatment with UA significantly inhibited phosphorylation of NF- κ B P65 compared to treatment with TNF- α alone (Figure 3F). Furthermore,

immunofluorescence detection demonstrated that UA effectively suppressed nuclear translocation of NF- κ B P65 induced by TNF- α (Figure 3 G). These findings suggested that UA might function as an inhibitor of TNF- α signaling by directly binding to TNF- α . Although we did not perform a direct TNF- α –TNFR1 binding competition assay, the observed inhibition of TNF- α -induced NF- κ B activation and nuclear translocation supported the functional consequence of UA–TNF- α interaction.

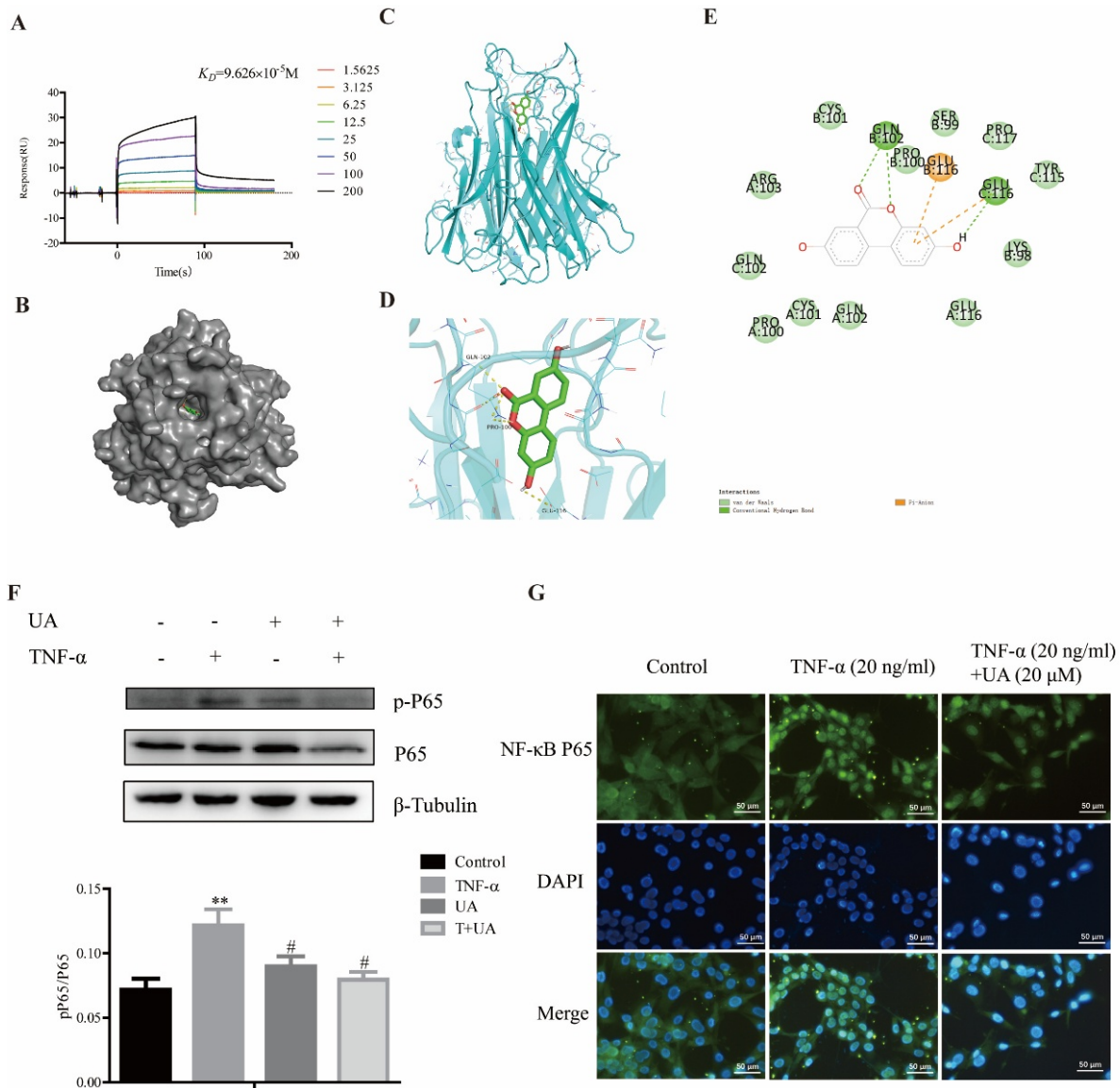
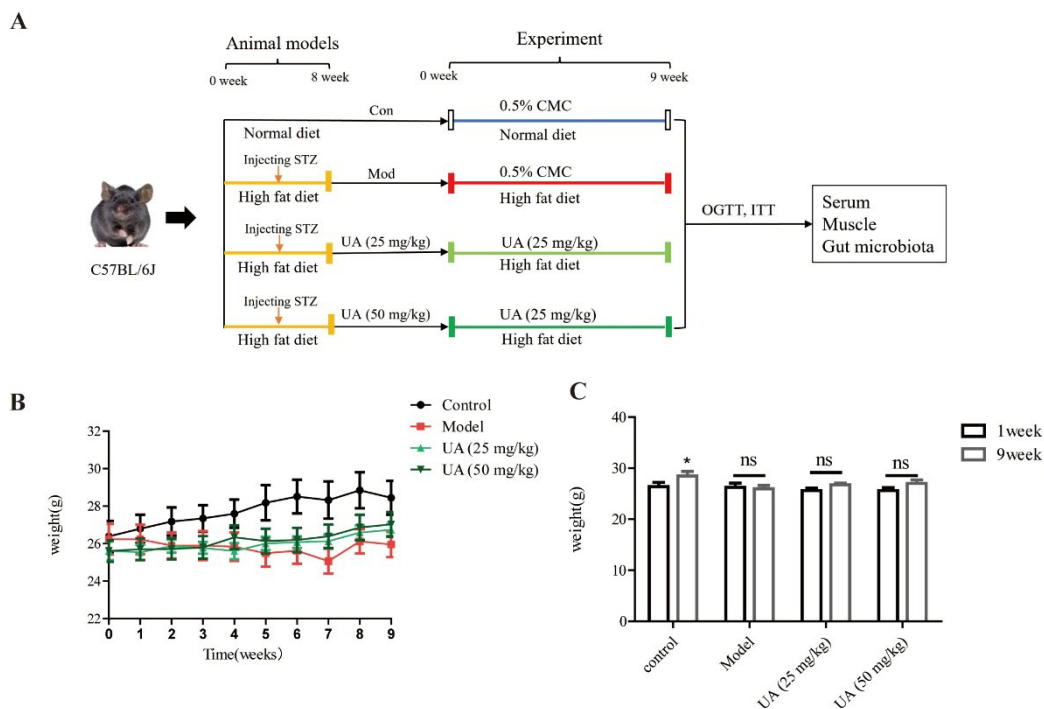


Figure 3. Direct binding of UA to TNF- α and inhibition of TNF- α -induced NF- κ B signaling. (A) Detection of the binding of UA and TNF- α by SPR method; (B) 3D images of UA docking TNF- α trimers and binding "pocket" positions; (C) three-dimensional docking image of UA docking TNF- α trimer, and (D) enlarged image of the junction; (E) 2D model diagram of UA binding to TNF- α trimer; (F) After 3T3-L1 cells were treated with TNF- α and UA separately or in combination for 20 min, the expression of pP65 and P65 proteins was detected by WB technology; (G) The nuclear translocation of P65 protein in 3T3-L1 cells treated with TNF- α and UA separately or in combination for 20 min was detected by cellular immunofluorescence technique. The values were means $\pm$ SEM ($n = 3$), compared with the Control group, ** $P < 0.01$; Compared with TNF- α group, # $P < 0.05$.

2.4 UA improves blood glucose levels in HFD-STZ-induced T2DM mice

In vitro evidence indicates that UA binds directly TNF- α , thereby ameliorating TNF- α -induced insulin resistance. To further explore the *in vivo* anti-diabetic activity of UA, We established a type 2 diabetes model characterized by high TNF- α expression by combining a high-fat diet (HFD) with streptozotocin (STZ)

administration. [27]. Throughout the animal experiment, all four groups of mice showed no significant difference in food and water intake. The mice were orally administered UA for 9 consecutive weeks, and their body weights were recorded weekly, and blood glucose levels were measured every two weeks (after fasting for 4 hours) (Figure 4 A). During the administration period, UA treatment showed a tendency to increase the body weight in T2DM mice compared with the Model group, although the difference was not statistically significant (Figure 4 B, C). Monitoring of blood glucose levels during the administration period revealed that both low dose (25 mg/kg) and high dose (50 mg/kg) of UA administration significantly reduced blood glucose levels in T2DM mice, no statistically significant difference was observed between high and low doses of UA treatments (Figure 4 D). Fasting blood glucose levels were also measured at the 9th week, which suggested that the fasting blood glucose levels were significantly decreased in both high and low doses of UA treated mice compared with the Model group (Figure 4 E). The insulin tolerance test (ITT) and oral glucose tolerance test (OGTT) are essential methods for evaluating pancreatic beta cell function and the organism's capability to regulate blood glucose in diabetic patients [28]. Therefore, ITT and OGTT were conducted to assess the therapeutic effect of UA on diabetic mice. Subcutaneous injection of 0.75 U insulin per mouse revealed a significant impairment in insulin tolerance in the Model group compared to the Control group; however, both low-dose and high-dose UA treatments significantly improved the insulin sensitivities in diabetic mice (Figure 4 F, G). For OGTT, mice were orally administered 1 g/kg glucose. It was observed that glucose tolerance was significantly impaired in the Model group compared to the Control group; nevertheless, both low-dose and high-dose UA treatments significantly improved glucose tolerance in diabetic mice (Figure 4 H, I). These findings indicate that UA treatments effectively reduce blood glucose levels and enhance blood glucose control and monitoring in T2DM mice.



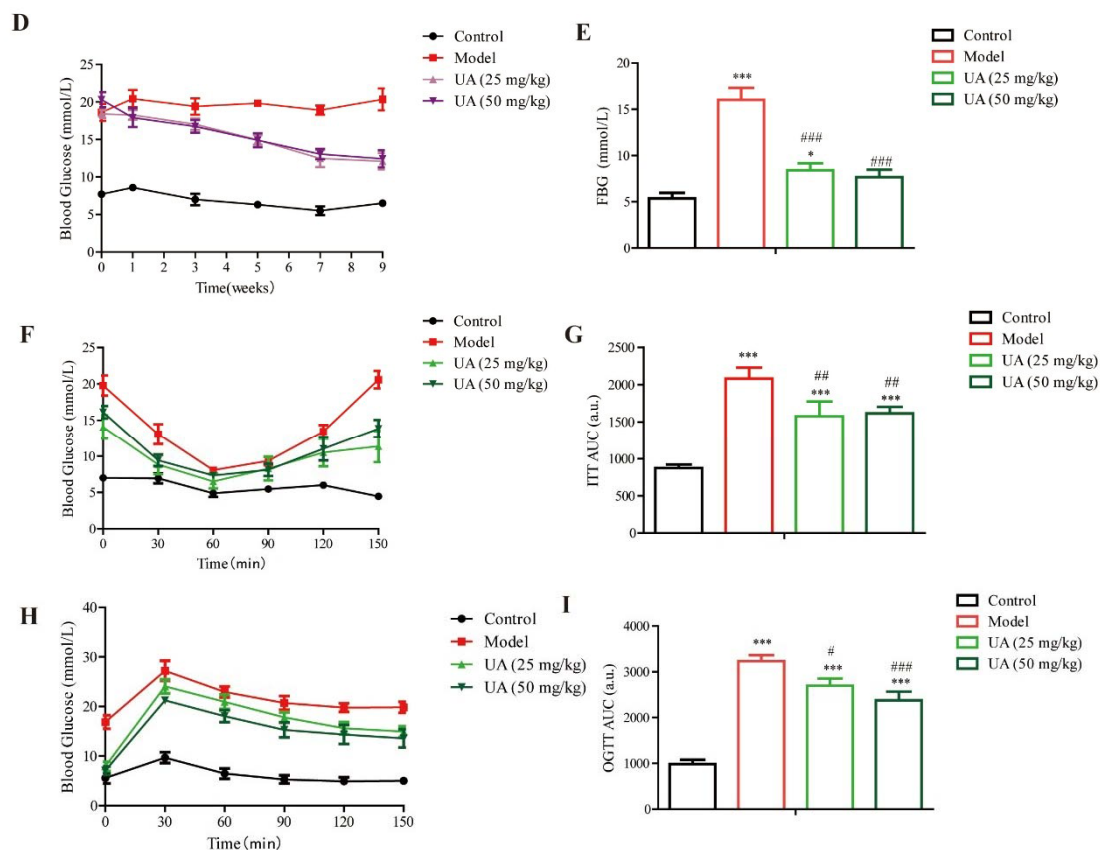


Figure 4. UA improves glucose homeostasis in T2DM mice. (A) Animal Experiment Flow Chart; (B) The weight changes of each group of mice each week during the experimental period; (C) Body weight of mice in each group at week 1 and week 9; (D) The weekly blood glucose changes during UA treatment; (E) Fasting blood glucose levels at the 9th week of administration; (F) Insulin tolerance test with 0.75 U/kg insulin injection was used to detect the blood glucose levels of each group of mice within 120 min, and (G) the area under the curve was calculated; (H) Glucose tolerance test was performed at the dose of 1 g/kg glucose to detect the blood glucose levels of each group of mice within 120 min, and (I) the area under the curve was calculated. The values were expressed by means $\pm$ SEM ($n \geq 6$), and in FIG. C, compared with control-1 week group, $*P < 0.05$. In Figure D-H, compared with the Control group, $*P < 0.05$, $***P < 0.001$; Compared with Model group, $\#P < 0.05$, $###P < 0.01$, $####P < 0.001$.

2.5 The effects of UA on serum indicators and diabetic complications in T2DM mice

To further investigate the impact of UA administration on serum insulin levels and inflammation-related key cytokines in type 2 diabetes, we employed enzyme-linked immunosorbent assay (ELISA) to detect the concentrations of insulin, C-peptide, glucagon, and TNF- α in blood samples. The results demonstrated a significant increase in serum TNF- α levels was observed in the Model group compared with the Control group, however, treatment with UA significantly reduced the serum TNF- α levels (Figure 5A). The insulin and C-peptide concentrations were significantly elevated in the Model group compared with the Control group. Both high-dose and low-dose UA treatments notably decreased the insulin and C-peptide levels (Figures 5B, C). No statistically significant difference was observed in glucagon levels across all groups (Figure 5D). Insulin is secreted by pancreatic β cells, and in T2DM, the interplay between hyperinsulinemia and hyperglycemia leads to pancreatic β cell atrophy [29]. Consequently, the restoration of insulin levels and the amelioration of the diabetic complications serve as indicators for T2DM improvement. By employing hematoxylin and eosin (HE) staining on pancreatic, renal, and hepatic tissues from all groups of mice, we observed that compared with the Control group, mice in the Model group exhibited characteristic features of

T2DM such as glomerular enlargement, fatty liver deposition, and pancreatic atrophy. Both high-dose and low-dose UA treatments effectively mitigated these diabetic complications (Figure 5E). These findings indicate that UA treatment significantly on reduce serum TNF- α levels and improve insulin secretion in T2DM mice. Moreover, it also exerts notable efficacy in alleviating diabetic complications.

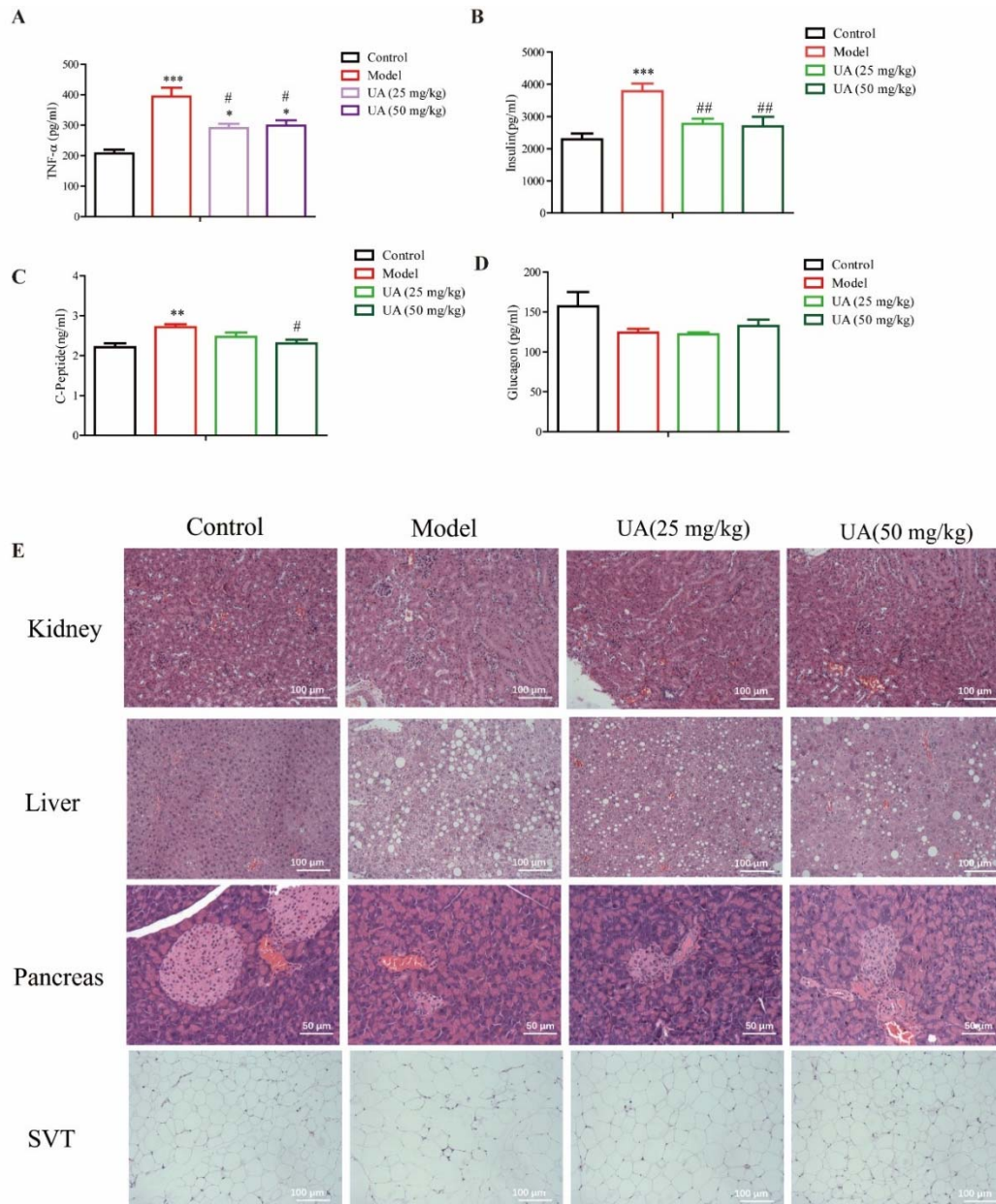


Figure 5. UA ameliorates serum TNF- α levels and tissue pathology in T2DM mice. (A) Expression of serum TNF- α in each group of mice; (B) The serum insulin concentrations in each group of mice; (C) serum C-peptide concentrations in each group of mice; (D) Serum glucagon concentrations of mice in each group. (E) HE staining was performed on the kidney, liver, pancreas and SVT tissues of mice in each group, and images were collected by inverted microscope. Values are expressed by means $\pm$ SEM ($n \geq 6$), compared with Control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Compared with Model, # $P < 0.05$, ## $P < 0.01$.

2.6 The effect of UA on insulin signaling pathway, glycogen synthesis genes, and inflammation-related gene expression in T2DM mice

To examine the effect of UA on the expression of key proteins in the insulin signaling pathway, Western blotting was employed to assess the expression levels of relevant proteins in muscle tissues. The results showed a significant increase in PTEN expression in mice from the Model group compared with the Control

group. However, treatment with low-dose and high-dose UA significantly reduced PTEN expression levels. Conversely, pAKT^{Ser473} expression levels showed an opposite trend to PTEN expression in all groups of mice [30]. Glut4 protein, a crucial player in cellular glucose uptake, showed a significant decrease in the Model group, UA treatment significantly restored its expression (Figure 6A). Analysis of inflammation and fatty acid synthesis-related genes revealed elevated expressions of *tnf-α*, *fasn*, and *acc1* in liver tissues of the Model group; treatment with a high-dose of UA effectively reduced their expressions (Figure 6B-D). In terms of glycogen synthesis-related genes determined in muscle tissues, expressions of *gys1*, *gsk3β* and *ugp2* were significantly reduced in Model group compared with Control group; nevertheless, treatment with a high-dose of UA restored their expressions (Figure 6E-G). These results indicated that UA treatment exerted a notable improvement effect on both key protein expressions involved in insulin signaling pathway as well as glycogen synthesis-related genes in muscle tissues of T2DM mice, while inflammation and fatty acid synthesis-related gene expressions were markedly reduced in liver tissues.

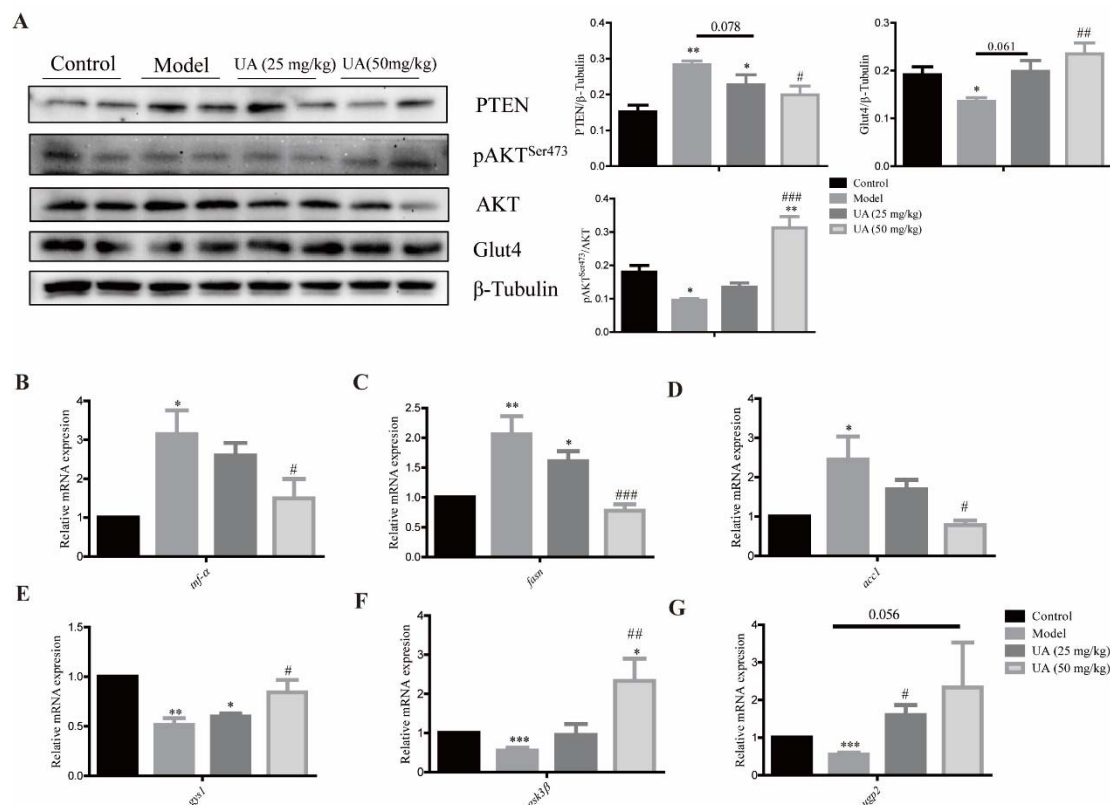


Figure 6. UA treatment increased insulin signaling and glycogen synthesis in muscle, and reduces inflammatory gene expression in liver. (A) Western Blot assay was used to detect the protein expression levels of PTEN, pAKT^{Ser473}, AKT and Glut4 in muscle tissue of mice in each group, and Image J was used for gray analysis of each band. (B) Expression of *tnf-α* mRNA, (C) *fasn* mRNA, (D) *acc1* mRNA in liver tissues of mice in each group; (E) *gys1* mRNA expression in muscle tissue of mice in each group, (F) *gsk3β* mRNA expression, (G) *ugp2* mRNA expression. Values are expressed by means ± SEM (n ≥ 4), compared with Control, **P* < 0.05, ***P* < 0.01, ****P* < 0.001; Compared with Model group, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001.

2.7 The effect of UA on the polarization of RAW264.7 cells towards M1 (pro-inflammatory) macrophages

Macrophages are typically classified into M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes. The characteristic secretory profile of M1 macrophages includes pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β. It has been demonstrated that modulation of macrophage polarization is

crucial for ameliorating inflammation-induced diseases^[31]. RAW264.7 cells, a murine monocyte/macrophage leukemia cell line, are extensively used in studies to investigate macrophage polarization. Previous studies have established that lipopolysaccharide (LPS) treatment can induce the polarization of RAW264.7 macrophages toward the M1 phenotype, a state characterizes by markers such as iNOS and COX-2^[32]. To further investigate whether UA treatment affects TNF- α production, we used RAW264.7 macrophages to determine the impact of UA on LPS-induced M1 polarization. Our results showed that LPS treatment significantly upregulated iNOS and COX-2 expression after 12 hours compared with the Control group; however, co-treatment with UA markedly suppressed LPS-induced elevated expression of iNOS and COX-2 (Figure 7A). Immunofluorescence staining demonstrated that LPS significantly increased iNOS expression which was effectively attenuated by UA treatment (Figure 7B). These results suggest that UA can inhibit LPS-induced polarization towards the M1 phenotype in macrophages.

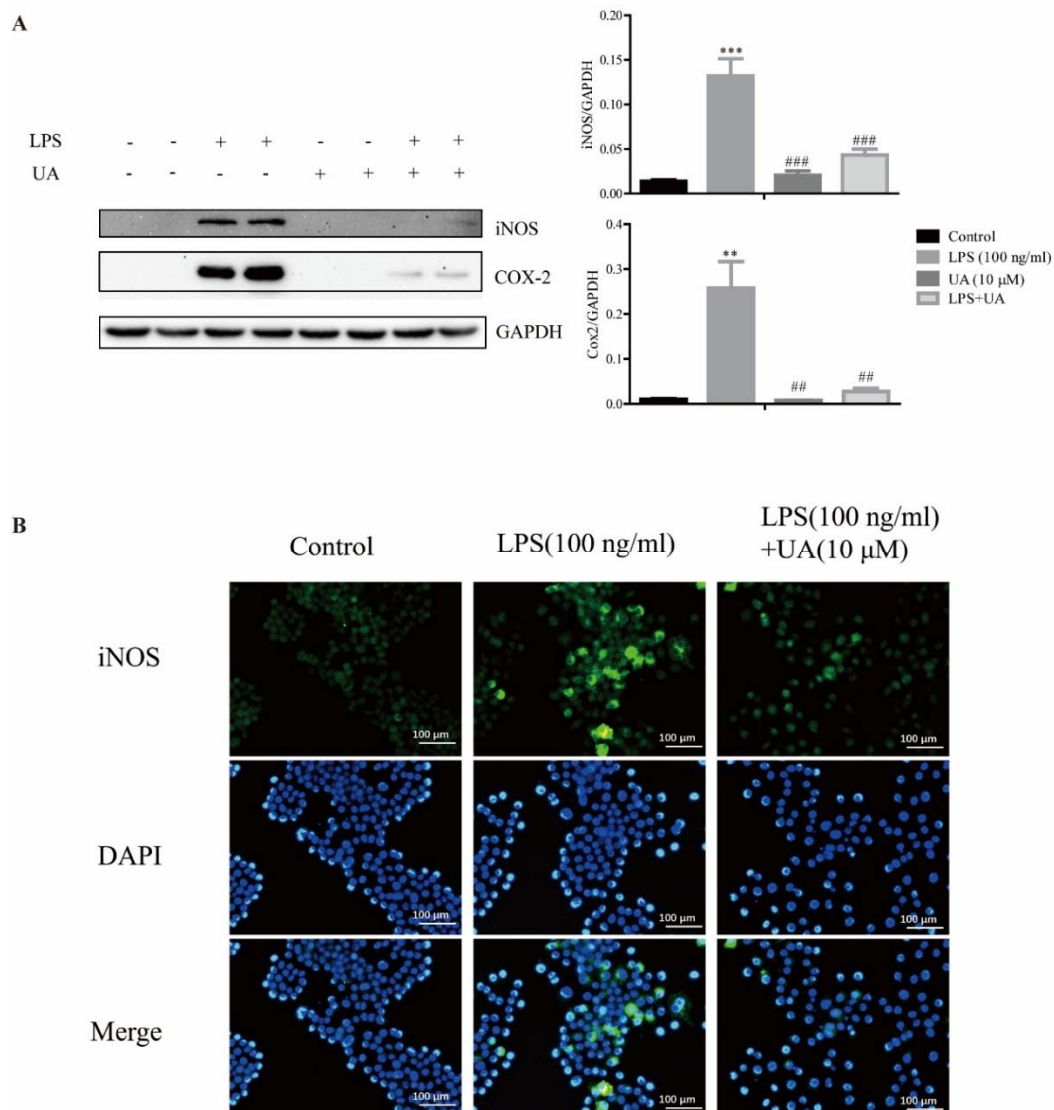
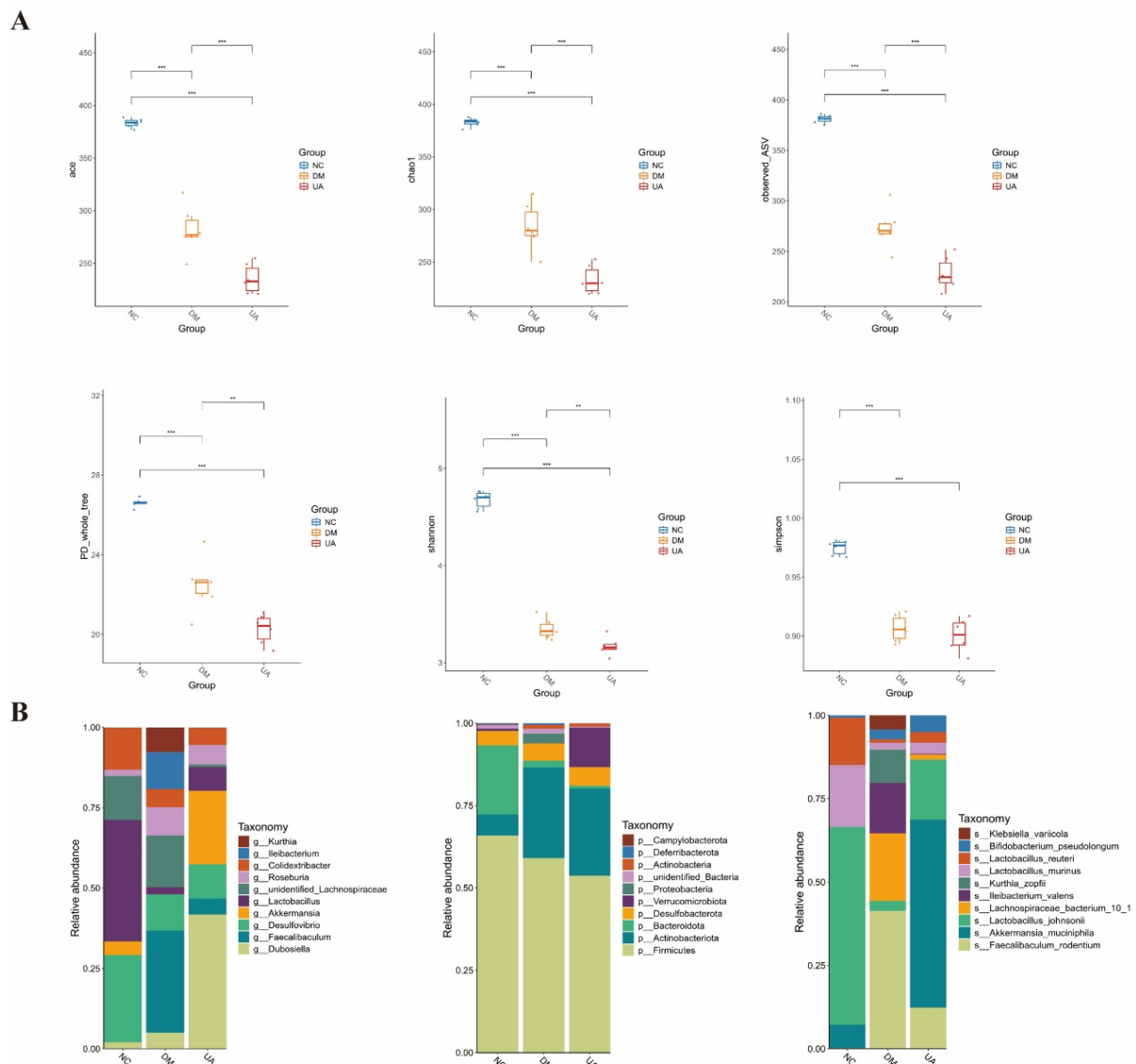


Figure 7. UA suppresses LPS-induced M1 polarization in RAW264.7 macrophages. (A) Western Blot assay was used to detect the protein expression levels of iNOS, COX2 after treatment with LPS (100 ng/ml), UA (10 μ M) and combination of LPS and UA in RAW264.7 cells, Image J was used for gray analysis of each band. (B) Following treatment of RAW264.7 cells with LPS (100 nM) and UA (10 μ M), either alone or in combination, iNOS protein levels were assessed via immunofluorescence. In the resulting images, iNOS is shown in green and nuclei are shown in blue. Images were acquired and recorded using an inverted fluorescence microscope. The analytical values were expressed by means $\pm$ SEM ($n \geq 4$), compared with Control, $**P < 0.01$, $***P < 0.001$; Compared with LPS group, $\#P < 0.05$, $\#\#P < 0.01$.

2.8 The effect of UA on the intestinal microbiota structure of T2DM mice

To explore the potential impact of UA on the intestinal ecosystem, we analyzed the microbial community structure in the fecal samples. The microbial community composition of the Model group mice was significantly different from that of the Control group, indicating that HFD/STZ induced a significant dysbiosis. Notably, the community cluster composition of the UA treatment group showed significantly greater uniformity, suggesting that the UA intervention effectively adjusted intestinal microbiota profile (Figure 8A). Additionally, the microbial composition at the phylum level showed that UA treatment significantly changed the dominant phyla, particularly reducing the elevated ratio of Firmicutes/Bacteroidetes in the Model group. Crucially, at the genus level, UA treatment significantly increased the relative abundance of several beneficial genera, including the beneficial bacteria *Akkermansia*, *Dubosiella* and *Kurthia*, etc. At the species level, UA treatment significantly increased the prominence of the key species *Akkermansia muciniphila* (Figure 8B). The heatmap of the most abundant features at the species level further confirmed these restorative changes (Figure 8C). These results indicate that the UA treatment can also improve the intestinal microbiota imbalance caused by HFD/STZ and increase the abundance of beneficial intestinal bacteria that improve the development of T2DM.



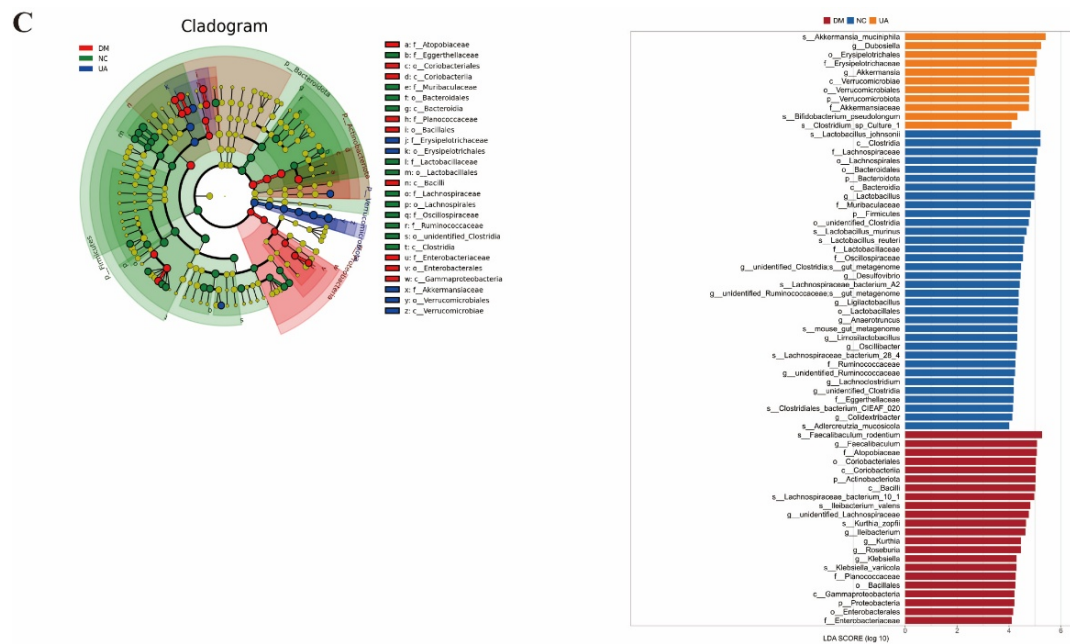


Figure 8. UA restores gut microbiota structure and enriches beneficial taxa in T2DM mice. (A) Species diversity and species richness. (B) Bacterial classification and quantitative analysis at the phylum, genus and species levels of the intestinal microbiota in different experimental groups. (C) LEfSe analysis of differential microbial (bacterial) populations. Significant differences in species between taxa with LDA values greater than 3. microbiomic characterization (n = 6). * $P < 0.05$, ** $P < .01$, *** $P < .001$

2.9 Functional Prediction of Gut Microbiota and Its Association with Host Metabolism

To elucidate the functional significance of the gut microbiota alterations induced by UA and their potential link to improved host metabolic phenotypes, we conducted functional prediction and correlation analysis based on 16S rRNA sequencing data. PICRUSt2 prediction against the KEGG database indicated that UA treatment significantly reshaped the functional potential of the gut microbiota, with a dominant enrichment in Carbohydrate Metabolism (Figure 9A), suggesting UA may influence host energy homeostasis by modulating microbial carbon utilization. Specifically, differential pathway analysis using the MetaCyc database revealed that key pathways within carbohydrate metabolism, including Glycolysis (PWY-5484) and the Pentose Phosphate Pathway (PENTOSE-P-PWY), were significantly upregulated in the UA-treated group (Figure 9B). This activation of core energy-metabolizing pathways was closely associated with beneficial bacteria enriched by UA, such as *Akkermansia*, indicating that specific taxa may drive community-level functional shifts. Furthermore, Spearman correlation analysis established a direct statistical link between microbiota changes and host clinical parameters. The results demonstrated a strong negative correlation between the relative abundance of *Akkermansia muciniphila*—a signature species enriched by UA—and serum levels of the key pro-inflammatory cytokine TNF- α (Figure 9C), thereby connecting UA-induced expansion of beneficial bacteria with the amelioration of systemic inflammation and insulin resistance.



Figure 9. Functional prediction of gut microbiota and its association with host metabolism after UA intervention. (A) Prediction of the relative abundance of the overall microbial community function. (B) (C) Comparison of the predicted abundance of representative carbohydrate metabolism pathways in the MetaCyc database between the two groups. (D) Heatmap of the correlation between key differential genera and host metabolic parameters. Red and green denote positive and negative correlations, respectively. * $P < 0.05$, ** $P < 0.01$.

3. Discussion

Type 2 diabetes is a chronic metabolic disorder that may present asymptomatic or mild symptoms in its early stages. This often leads to underestimation and delayed diagnosis, which is closely linked to its rapid progression [33]. As our understanding of diabetes advances and the number of diabetic patients increase, it has become evident that low-grade inflammation and immune system activation play crucial roles in

obesity-related insulin resistance and the pathogenesis of type 2 diabetes. Therefore, inhibiting inflammatory responses can serve as an effective therapeutic strategy for improving T2DM [34]. Numerous studies have demonstrated the significant role of dietary polyphenols such as apigenin, quercetin, kaempferol, curcumin, resveratrol, and ellagic acid in regulating inflammation [35], while also exhibiting positive effects on T2DM management [36]. UA, which serves as the primary intestinal metabolite of ellagic acid, belongs to a class of hydroxybenzoquinone compounds possessing anti-inflammatory properties [37], antioxidant activity [38], anticancer potential [39], and antidiabetic effects [40]. Xiao Y et al. demonstrated that UA exerts beneficial effects on diabetes-related cognitive impairment by enhancing intestinal barrier function and reducing circulating levels of metabolic endotoxins and pro-inflammatory cytokines [41]. Moreover, UA exhibits anti-inflammatory and anti-diabetic properties by suppressing pancreatic inflammation in HFD+STZ-induced diabetic mice through activation of AMPK signaling pathway and induction of autophagy [42]. These findings provide compelling evidence for the therapeutic potential of UA for mitigating diabetes-associated inflammation. This aligns with the emerging recognition that natural products often exert benefits through multi-target mechanisms, as seen with other bioactive compounds like GPAGAP, FER1L6, and D-mannose [43-45].

As an inflammatory factor, TNF- α has been established as a pivotal contributor to the development of insulin resistance [46], and inhibition of TNF- α action has demonstrated efficacy in ameliorating insulin resistance [47]. Monoclonal antibody drugs targeting TNF- α , such as infliximab and adalimumab, used for rheumatoid arthritis treatment have shown significant reductions in blood glucose levels [26, 47]. Several natural compounds, such as Baicalin and *Biancaea decapetala* (Roth) O.Deg. extract, have been reported to directly bind to inflammatory cytokines or their receptors, thereby inhibiting downstream signaling [48, 49]. In line with these findings, our SPR and docking data suggest that UA may operate through a similar direct-targeting mechanism. This study has certain limitations that warrant discussion. First, the animal experiment employed two effective doses of UA (25 and 50 mg/kg). While both doses yielded significant metabolic improvements, the lack of a full dose-response relationship, as neither a lower sub-effective dose nor a potential higher plateau dose were determined, limits our ability to precisely delineate the therapeutic window. Future studies utilizing an expanded dose range will be essential to fully establish a complete pharmacokinetic-pharmacodynamic profile. Second, only male mice were used in this study, which was to avoid the potential confounding effects of the female estrous cycle on metabolic and inflammatory parameters, a standard approach in initial metabolic phenotyping studies to reduce inter-individual variability. We acknowledge that investigating potential sex differences in UA's efficacy represents an important future direction. Lastly, regarding the *in vitro* observation of reduced C2C12 viability at 20 μ M, we emphasize that the cell survival rate remained > 90%, which is considered acceptable for functional pharmacological assays, ensuring that the observed improvements in insulin signaling were due to UA's bioactivity rather than cytotoxicity. In this study, TNF- α -induced insulin resistance model was used to investigate whether UA functioned similarly to a TNF- α monoclonal antibody through binding to TNF- α and blocking its effects. Our

findings revealed that UA treatment restored TNF- α -induced insulin resistance, as demonstrated by a notable recovery of glucose uptake, restoration of the insulin signaling pathway, and increased Glut4 membrane translocation. These results highlight the substantial recovery effect of UA on TNF- α -mediated insulin resistance. The binding affinity and mode of interaction between UA and TNF- α were further confirmed using SPR technology and computer molecular docking techniques. It was revealed that UA exhibited a high binding affinity towards TNF- α (9.626×10^{-5} M) with a corresponding binding energy of -8.1 kcal/mol, primarily through the formation of hydrogen bonds and hydrophobic interactions. These findings suggest that UA may effectively inhibit the binding of TNF- α to its receptor TNFR1, thereby exerting inhibitory effects on TNF- α -induced insulin resistance. The effectiveness of TNF- α 's action and activation of signaling pathways can be indicated by the phosphorylation and nuclear translocation of NF- κ B P65 [50, 51]. In short-term experiments, it was observed that UA significantly suppressed both the expression and nuclear translocation of pP65 induced by TNF- α , providing further evidence for its capacity to block downstream signaling pathways activated by TNF- α . It should be noted that while SPR and molecular docking demonstrate direct binding of UA to TNF- α , and downstream signaling assays show inhibition of TNF- α -induced NF- κ B activation, we did not provide direct evidence that UA blocks TNF- α binding to TNFR1. Future studies employing competitive binding assays or receptor interaction assays would be valuable to further validate the mechanistic details of UA as a TNF- α -targeting compound.

The present study confirmed *in vitro* that UA ameliorates insulin resistance by directly binding to and inhibiting TNF- α . To validate its therapeutic effect *in vivo*, we employed an HFD/STZ-induced T2DM mouse model characterized by high TNF- α expression [52, 53]. Oral administration of UA significantly reduced blood glucose levels and improved glucose tolerance and insulin sensitivity, as evidenced by OGTT and ITT tests, without significantly altering body weight (Fig. 4). UA also improved key serum biomarkers, reducing elevated levels of TNF- α , insulin, and C-peptide [54, 55]. Furthermore, UA intervention effectively ameliorated HFD/STZ-induced gut dysbiosis, reduced the Firmicutes/Bacteroidetes ratio and increased beneficial genera such as *Akkermansia* and *Dubosiella*, including the key species *Akkermansia muciniphila*. Importantly, functional prediction analysis further revealed that this restorative shift was characterized by an enrichment in microbial carbohydrate metabolism pathways (Fig. 9A, B). Crucially, a strong negative correlation was observed between the abundance of *Akkermansia muciniphila* and systemic TNF- α levels (Fig. 9C), providing a direct statistical link between a specific microbial change and the amelioration of systemic inflammation. This restoration of gut microbial ecology likely represents an additional mechanism alleviating systemic inflammation. Consistently, UA attenuated hepatic expression of *tnf- α* and fatty acid synthesis genes (*fasn*, *acc*), and ameliorated T2DM complications in the pancreas, liver, and kidney. At the molecular level in muscle tissue, UA enhanced the insulin signaling pathway (suppressed PTEN, increased pAKT^{Ser473} and Glut4) and upregulated glycogen synthesis genes (*Gys1*, *Gsk3 β* , *Ugp2*). These findings indicate a pronounced ameliorative effect of UA on the insulin signaling pathway in T2DM mice. Additionally, inflammation and lipid accumulation play pivotal roles in the pathogenesis of T2DM [56]. It is noteworthy that despite these

comprehensive metabolic improvements, UA did not induce significant weight loss in T2DM model. This observation contrasts with reports that UA enhances thermogenesis in adipose tissue^[19]. We propose several explanations for this discrepancy. First, the primary defect in our HFD/STZ model is severe insulin resistance. UA's potent effect on improving insulin signaling and glucose disposal may have been the dominant metabolic benefit under continued high-fat feeding, which does not necessarily equate to weight loss. Second, thermogenesis studies often focus on energy expenditure in obesity models, whereas our study prioritized resolving glucose intolerance and inflammation in T2DM mice. Differences in dose, duration, or metabolic context (diabetes vs. obesity) may also influence the results. Thus, in the setting of T2DM, UA's beneficial effects appear more closely linked to restoring metabolic flexibility and insulin action rather than altering overall energy balance.

Macrophage polarization refers to the process by which macrophages are polarized into M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes under the influence of various factors^[57]. It has been demonstrated that UA can attenuate macrophage polarization towards the M1 phenotype through upregulation of autophagy flux in macrophages^[16], indicating its potential to modulate inflammatory responses and suppress inflammation. In our study, LPS treatment significantly promoted the expression of M1-type macrophage markers iNOS and COX-2. While treatment with UA effectively inhibited the enhanced expression of iNOS and COX-2 induced by LPS. These results indicate that UA has the ability to prevent macrophages from polarizing towards the M1 phenotype, thereby reducing the release of pro-inflammatory cytokines.

Previous studies have already demonstrated that UA has multiple biological activities. This study reveals that UA improves T2DM through a multi-pronged mechanism involving direct TNF- α interaction, anti-inflammatory macrophage polarization, and gut microbiota restoration. An important question arising from these parallel findings is the causal relationship and potential hierarchy among these effects. Regarding the interplay between microbiota modulation and systemic inflammation—a classic “chicken-and-egg” dilemma in microbiome research—our observations primarily reflect a correlation. The precise causal direction cannot be definitively resolved by our current experimental design. We consider a bidirectional interaction model to be most plausible. On one hand, UA-induced enrichment of beneficial bacteria like *Akkermansia muciniphila*, which is strongly negatively correlated with serum TNF- α (Fig. 9C), may enhance gut barrier integrity and reduce the translocation of pro-inflammatory molecules (e.g., LPS), thereby dampening systemic inflammation. On the other hand, the amelioration of the systemic pro-inflammatory milieu by UA (via direct TNF- α targeting and macrophage modulation) could in turn foster a gut environment more favorable for the growth of beneficial commensals. Thus, UA-mediated improvement in T2DM likely involves a positive feedback loop where direct anti-inflammatory effects and microbiota restoration reinforce each other.

Considering the temporal and spatial logic of these actions, we can further hypothesize a potential mechanistic hierarchy. The direct targeting of TNF- α and immune cells, evidenced by *in vitro*

binding and rapid signaling inhibition occurring within minutes to hours (Figs. 2 & 3), likely represents a primary and rapid initiating event. The modulation of the gut microbial community structure and function, observed after weeks of intervention *in vivo* (Figs. 8 & 9), may then act as a secondary crucial consolidating mechanism that sustains metabolic benefits. Therefore, UA's therapeutic efficacy likely stems from an integrated sequence of events: initial anti-inflammatory signaling blockade, followed by synergistic restoration of host-microbe homeostasis. Future studies employing fecal microbiota transplantation (FMT) will be crucial to disentangle the causality and establish the sufficiency of the UA-modulated microbiota.

4. Conclusion

In summary, our findings in this study demonstrate that UA effectively inhibits the effects of TNF- α by binding to it *in vitro*, thereby ameliorating TNF- α -induced insulin resistance. Moreover, owing to its diverse biological activities, UA effectively inhibits the M1 - type polarization of macrophages induced by LPS and significantly reduces the secretion of TNF- α . Additionally, UA intervention significantly ameliorated HFD/STZ-induced intestinal microbiota dysbiosis by reducing the Firmicutes/Bacteroidetes ratio and increasing the abundance of beneficial bacteria, including *Akkermansia* and *Dubosiella*, which likely contributes to the attenuation of systemic inflammation and improvement of metabolic homeostasis. Although direct evidence from *in vivo* experiments supporting the improvement of insulin resistance by UA through inhibition of TNF- α effects is lacking, it significantly reduces serum levels of TNF- α and insulin in T2DM mice and inhibits PTEN protein expression in muscle tissue. These results suggest that UA exerts an inhibitory effect on both the production and action of TNF- α within the body. This dual-action profile functionally parallels that of therapeutic anti-TNF- α monoclonal antibodies, positioning UA as a natural product-based strategy for alleviating insulin resistance *in vivo* and *in vitro* (Figure 10).

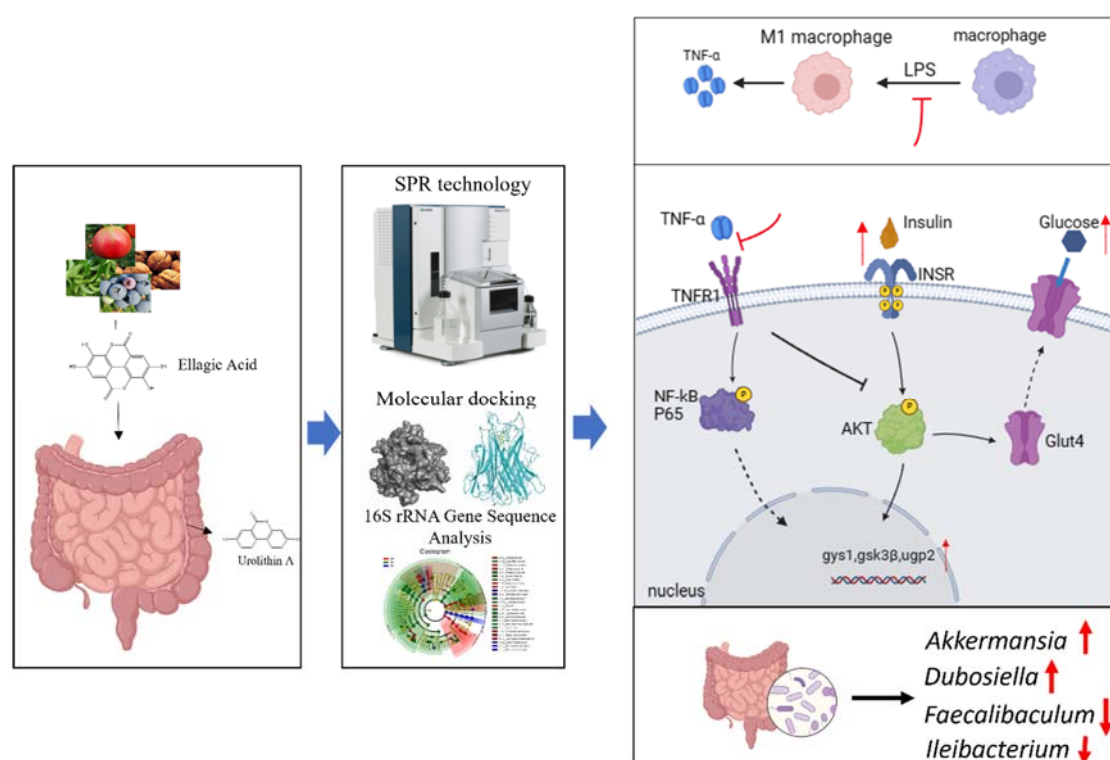


Figure 10. In this study, combining SPR technology, molecular docking technology, in vitro experiments and in vivo experiments, it was demonstrated that UA can block the effects of TNF- α by binding to it *in vitro*, thereby alleviating TNF- α -induced insulin resistance. UA suppresses LPS-induced M1 polarization of macrophages. Furthermore, UA administration significantly ameliorated intestinal microbiota dysbiosis in T2DM mice by reducing the Firmicutes/Bacteroidetes ratio and enriching beneficial bacteria such as *Akkermansia muciniphila*, which represented an additional mechanism contributing to the alleviation of systemic inflammation and metabolic improvement. *In vivo* experiments showed that UA significantly reduced serum levels of insulin and TNF- α in T2DM mice and enhanced insulin signal transduction in muscle tissue, indicating that UA has inhibitory effects on the production and actions of TNF- α in vivo. Image created with Biobender.com.

5. Materials and Methods

5.1 Cell culture

C2C12 mouse myoblasts, 3T3-L1 mouse adipocytes, and RAW264.7 mouse monocyte macrophages were procured from the Cell Bank of Kunming Institute of Zoology, Chinese Academy of Sciences. All cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), and incubated at a temperature of 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air. Prior to subsequent experiments, C2C12 cells underwent differentiation by replacing the culture medium with DMEM supplemented with 2% horse serum upon reaching approximately 90% confluence in the culture dish; this process was repeated every two days for medium replenishment. After seven days of differentiation, further experimental procedures were conducted.

5.2 Western blot

The cellular and animal tissue proteins were extracted using RIPA buffer with a concentration of 1mM. The protein concentration was determined using the BCA method. Subsequently, the proteins were separated on an 8% SDS-PAGE gel and transferred onto a PVDF membrane with a pore size of 0.45 μ m. After blocking with 5% skim milk for 1 hour, incubation with primary antibodies including pIR^{Tyr1150} #3024, IR#3025,

pAKT^{ser473}#9271, AKT#9272, Glut4#2213, NF- κ B P65#8242, pNF- κ B P65#3033, PTEN#9552 purchased from CST was carried out at 4°C for 16-20 hours. This was followed by incubation with corresponding secondary antibodies at room temperature for 1 hour. Finally, the protein bands were detected using ECL reagent.

5.3 RNA extraction and real-time fluorescence quantitative PCR

The Trizol method (Takara) was employed for the extraction of total RNA from cells. Reverse transcription was performed using 1000ng of total RNA and the PrimeScript RT reagent Kit (Takara). Quantitative real-time PCR analysis was conducted on a Roche LC480 instrument with TB Green Premix Ex Taq II (Takara). The mRNA quantity was determined using the $\Delta\Delta C_t$ method. The primer sequences utilized are provided in Table 1.

Table 1 Real-time PCR primers.

target gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>β-actin</i>	GAGACCTTCAACACCCAGC	ATGTCACGCACGATTTCCC
<i>fasn</i>	CTGGCATTCTGATGGAGTC	TGTTTCCCCTGAGCCATGTA
<i>acc1</i>	GGCTATGCTGCGCTTAACA	GCCTCTGTTTTGTGGGTGAC
<i>gys1</i>	CTTCCTCATCATAACGCTCAC	CTTTCCAGACCACTTCACCTA
<i>gsk3β</i>	CAATTCAGCCAACACACAGC	TCCATTCTTTGGAATCTGC
<i>ugp2</i>	TAACCAAGGGCACTGTAGGGA	GGAGCTGCAATTAAGTTTCG
<i>tnf-α</i>	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG

5.4 Immunocytochemistry (ICC)

After drug treatment, the cells were fixed in 4% paraformaldehyde for 20 minutes, followed by blocking with a solution of 1% BSA and 0.5% TritonX-100 at room temperature for 1.5 hours. The primary antibodies (iNOS #A0312, NF- κ B P65 #A2547, and Glut4 #A7637) purchased from abclonal company were incubated with the cells at 4°C for a duration of 16-20 hours. Subsequently, corresponding secondary antibodies specific to the appropriate species were incubated with the cells at 4°C for a period of 4 hours. Finally, the cells were sealed in Antifade mount Medium containing DAPI and observed under a fluorescence microscope.

5.5 Glucose uptake experiment

2-NBDG, a fluorescently labeled D-glucose analogue, is employed to evaluate glucose uptake capacity of viable cells [58]. Upon cellular entry, 2-NBDG undergoes phosphorylation by hexokinase and becomes non-degradable, remaining intracellularly. The attached fluorescent group can be quantified using flow cytometry, with fluorescence intensity serving as an indicator of cellular glucose uptake ability. Initially, the culture medium is substituted with FBS- and D-glucose-free DMEM medium. Subsequently, both 2-NBDG and drugs are simultaneously introduced into the medium. Following treatment, intracellular fluorescence intensity is measured using flow cytometry and data analysis is performed utilizing FLOWJ V10 software.

5.6 Surface Plasmon Resonance (SPR) experiment

Surface plasmon resonance (SPR) is employed for the detection of protein-ligand interactions, including those involving proteins [59]. The Biacore S200 system was utilized to investigate the binding between UA and

TNF- α . A CM5 chip was selected, onto which TNF- α was immobilized via amino coupling on its channels. The concentration gradient of UA ranged from 0 μ M to 200 μ M, with specific concentrations set at 1.5625 μ M, 3.125 μ M, 6.25 μ M, 12.5 μ M, 25 μ M, 50 μ M, 100 μ M, and finally reaching a maximum of 200 μ M. Molecular interaction experiments were conducted following the established protocol and analyzed using the Biacore S200 analysis software in order to obtain Sensorgram spectra for further analysis.

5.7 Molecular docking

Dock the TNF- α protein (1TNF) with UA by retrieving the protein structure from the RCSB PDB database (<http://www.rcsb.org>). Eliminate water molecules and ligands using PyMOL, followed by importing the target protein into AutoDock Tools 1.5.6 for hydrogenation treatment. Import UA as a ligand and conduct docking simulations with the target protein, saving in PDBQT format. Visualize the results using PyMOL and Discovery Studio 2019 Client.

5.8 Animal Experimentation

The animal experiments were conducted in accordance with the guidelines of the Animal Ethics Committee of Yunnan Agricultural University (approval number YUAN2023LLWYH003-5). Six-week-old male C57BL/6J mice were obtained from the Experimental Animal Center of Yunnan University. These mice were housed under controlled conditions at a temperature of $24 \pm 2^\circ\text{C}$ and a 12-hour light/dark cycle. They received a high-fat diet (60% fat, 20% protein, 20% carbohydrates) along with streptozotocin injection (40 mg/kg for three consecutive days) to induce a type 2 diabetes model^[60]. At the beginning of the experiment, the model mice were divided into four groups: Control group fed normal maintenance feed; Model group fed high-fat diet; low-dose UA group (UA 25mg/kg), administered oral gavage of UA solution at a dose of 25 mg/kg/day while being fed HFD; high-dose UA group (UA 50mg/kg), administered oral gavage of UA solution at a dose of 50 mg/kg/day while being fed HFD for nine weeks. The doses of UA (25 and 50 mg/kg/day) were selected based on previous studies demonstrating its efficacy in improving metabolic parameters in rodent models of obesity and diabetes^[61, 62]. The body weight and blood glucose levels of all four groups were monitored weekly. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed in the final week to assess blood sugar control and pancreatic function^[28]. After fasting for 12 hours before OGTT experiment, mice orally received glucose solution (2g/kg), and blood glucose levels at different time points were measured. For ITT experiment after fasting for two hours, mice received subcutaneous injection of insulin (0.75U/kg), and blood glucose levels at different time points were measured to calculate area under curve (AUC) representing changes in glucose level over time as an evaluation measure for mouse's glucose tolerance and insulin sensitivity abilities. Following the experimental procedure, euthanasia was conducted on the mice to collect serum samples for assessing levels of TNF- α , insulin, C-peptide, and glucagon using distinct Elisa kits. Additionally, muscle, adipose tissue, and liver tissue were procured for subsequent investigations.

5.9 16S rRNA Sequencing and Gut Microbiota Analysis

Fresh fecal samples were collected from mice at the end of the administration period and stored at -80°C . Microbial genomic DNA was extracted using the QIAamp PowerFecal Pro DNA Kit. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified and sequenced on an Illumina MiSeq platform. Sequencing data were processed using QIIME2. Operational Taxonomic Units (OTUs) were clustered with a 97% similarity threshold. Alpha diversity (Chao1, Shannon index) and Beta diversity (PCoA based on UniFrac distance) were analyzed. Linear discriminant analysis Effect Size (LEfSe) was used to identify differentially abundant taxa. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was employed to predict the functional potential of the gut microbiota.

5.10 H&E staining

The kidney, liver, and pancreas tissue samples from each group of mice were excised and immersed in a 10% formalin solution. Subsequently, they were dehydrated, embedded in paraffin, and cut into 5 μm thick sections. The cellular dimensions were assessed using HE staining. Finally, the sections were observed under appropriate magnifications of $40\times$ and $20\times$.

5.11 Data analysis.

Data are presented as the mean $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) and T tests were performed using the SPSS 22.0 software package. A value of $P < 0.05$ was considered to be statistically significant.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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