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Highlights

- Surfactin alleviates HFD/STZ-induced insulin resistance.
- Surfactin activates the AMPK/PI3K/Akt signalling pathways in the liver and skeletal muscle.
- Surfactin inactivates inflammatory pathways by MAPK/NF- κ B signaling pathways.
- Surfactin enhances the glucose uptake of skeletal muscle and adipose tissue.

**Surfactin alleviates insulin resistance by modulating the AMPK/PI3K/Akt signalling
pathway and inactivating inflammatory pathways**

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Author contributions

Chen Xiaoyu contributed to study design, preparation and drafting of the manuscript. Chen Xiaoyu, and Zhao Hongyuan contributed to data collection and analysis. Meng Fanqiang, Shenjuan, Tangchao, Lu Yingjian. and Lu Zhaoxin revised the manuscript.

Abstract: Type 2 diabetes mellitus (T2DM) is accompanied by insulin resistance in liver and peripheral tissue. *Bacillus amyloliquefaciens* fmb50 produces lipopeptide surfactin, which has a wide range of biological activities. However, the effects of surfactin on insulin resistance in any mouse model have not been reported. Our previous research demonstrated that dietary supplementation with surfactin can alleviate high fat diet (HFD)/ streptozotocin (STZ)-induced T2DM in mice by regulating intestinal microbiota. This study aims to explore the detailed mechanism of the effect of surfactin on liver and peripheral tissue. The results indicated that surfactin inhibited hepatic gluconeogenesis and promoted hepatic glycogen synthesis by activating the adenosine monophosphate-activated protein kinase (AMPK) and phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB/Akt) signalling pathways. Similarly, surfactin also significantly increased muscle glycogen synthesis by activating the PI3K/Akt signalling pathway. The glucose transporter 4 (GLUT4) levels of skeletal muscle and adipose tissue, which are insulin-mediated vital organs, was noticeably increased after surfactin treatment. In addition, our results indicate that decreased hepatic fat accumulation and suppressed inflammation and oxidative stress in liver, skeletal muscle and adipose tissue also play critical roles in ameliorating insulin resistance. These findings demonstrate that surfactin not only ameliorates liver dysfunction but also enhances insulin sensitivity in HFD/STZ-induced T2DM mice. Finally, using fasting blood glucose, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT), we show that early surfactin intervention can mitigate T2DM in mice. The study results warrant future functional food studies which will provide insight into diet-host interactions.

Keywords : T2DM, insulin resistance, GLUT4, glucose metabolism, AMPK/PI3K/Akt

1. Introduction

Insulin resistance (IR) in T2DM occurs as a result of a progressive decline in insulin action^[1]. Metabolic disorders, such as glucose transport, glycogen synthesis, gluconeogenesis and related protein synthesis often occur in T2DM patients. Insulin-stimulated glucose transport into adipose tissue of the epididymis and skeletal muscle is reduced in T2DM patients with IR^[2-3]. These are all independent mortality and morbidity risk promoters and a global socioeconomic burden^[4], and this has caused a great deal of global concern.

To date, common anti-diabetic drugs, metformin, is safe and cheap, but it often causes gastrointestinal side effects such as diarrhoea flatulence^[5]. Recommended insulin injection strategy also brings hypoglycaemia symptom^[3]. On the other hand, increasing evidence indicates that functional food and probiotics are a strategy for improving T2DM without side effects. Wu et al.^[2] found that the polysaccharide produced by *Vigna angularis* can activate insulin/PI3K/Akt pathways to relieve diabetes. *Dendrobium officinale* polysaccharide can alleviate IR by regulating AMPK/GLUT4 and promoting the translocation of GLUT4 in skeletal muscle and adipose tissue^[6]. Diabetic rats were administered by a *Bacillus*'s peptide that reduced blood glucose levels and α -amylase activity and reduced the damage to beta cells^[7]. Wang et al^[8] suggested that peptides extracted by casein hydrolysates also alleviate diabetes in rats via repairing hepatic oxidative damage. In diabetic mice and HepG2 cells, phenethyl caffeic acid can improve IR through inactivating the MAPK/JNK and NF- κ B pathway^[9]. Recently, surfactin was reported to relieve liver dysfunction through inhibiting proinflammation response^[10]. Despite this, little has been studied about how it controls glucose levels. Moreover, studies of surfactin's physiological toxicity have found that its oral median lethal dose (LD50) exceeded 2,500^[11] and 5,000 mg/kg,

demonstrating that surfactin is safe. Nevertheless, human health-related clinical research for peptides is still lacking^[12].

In our previous study, we demonstrated that surfactin ameliorated IR in HepG2 cells by regulating the PI3K/AMPK/GLUT4 signalling pathway^[13]. Our previous results also showed that dietary supplementation with surfactin in mice alleviated HFD/STZ-induced T2DM by modulating the intestinal microbiota and suppressing inflammation^[14]. Therefore, here, we used western blotting to explore surfactin's effects on glucose metabolism, oxidative stress, and inflammation-related proteins in T2DM mice in the liver, skeletal muscle and adipose tissue. Additional experiments were performed to further identify surfactin's preventive effect on mice with T2DM induced by HFD/STZ.

2. Materials and methods

2.1 Materials and reagents

The laboratory of food biotechnology, College of Food Science and Technology at Nanjing Agricultural University (Nanjing, China) provided a strain of *Bacillus amyloliquefaciens* fmb50 (fmb50)^[14]. Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) supplied STZ. Jiangsu Affinity Biosciences Co., Ltd. (Changzhou, China) supplied the primary antibodies against NF- κ B, tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, GLUT4, AMPK, phosphoinositide-AMPK (p-AMPK), PI3K, phosphoinositide-PI3K (p-PI3K), Akt, phosphoinositide-Akt (p-Akt), phosphoenolpyruvate carboxykinase (PEPCK), forkhead box protein 1 (FOXO1), glycogen synthesis (GS), glycogen synthase kinase-3 β (GSK-3 β), JNK, extracellular regulated protein kinases (ERK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Cell Signaling Technology Co., Ltd (Massachusetts, USA) supplied the primary

antibodies against phosphorylated-JNK (p-JNK), phosphorylated-ERK (p-ERK) and phosphorylated-NF- κ B (p-NF- κ B). Yeaman Chemical Industry (Nanjing, China) provided Gold Band 3-color Regular Range Protein Markers. GE Healthcare Life Science (Pittsburgh, USA) provided a nitrocellulose (NC) membrane. Beijing Solarbio Science & Technology Co., Ltd. provided 0.1% Tween 20 TBST. Beyotime Institute of Biotechnology, (Shanghai, China) provided HRP-linked anti-rabbit IgG secondary antibody. Affinity Biosciences (Changzhou, China) provided ECL plus solution. Vazyme Biotech (Nanjing, China) provided a one-step PAGE gel fast preparation kit (12%). Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) provided all other reagents.

2.2 Preparation of surfactin from *fmb50*

Surfactin was obtained from *B. amyloliquefaciens* fmb50 fermentation broth using a 50 L fermenter. Fermentation broths were flocculated using chitosan (0.5 g/L) and sodium alginate (0.3 g/L), adjusted pH to 5.0, and were filtered using a Buchner funnel with a filter cake of 0.5 cm thick diatomaceous earth, and then dissolved with absolute ethanol, and freeze-dried. A high-performance liquid chromatography was employed to detect surfactin sample^[15]. Approximately 88.6% pure of dried surfactin samples after purification was stored at 4 °C for further testing^[13-14].

2.3 Animal experimental design

Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) provided thirty healthy 4-week-old male Kunming mice. Experimental animals are licensed with the number SCXK (Jiansu) 2012-0004. A permit number NJAU. 2010317024, issued by the Ethics Committee, was required prior to any experiment and all experiments were performed following the Guide for

the Care and Use of Laboratory Animals. Researchers have previously described feeding environments and treatments^[16]. To acclimate the animals, all mice were kept in aseptic environments under constant conditions (25 °C; 12-hour light-dark cycle) for seven days before experiment. A random sampling of ten mice per group was then conducted, followed by randomization into three groups. Controls consumed basic diets for 12 weeks, while T2DM and surfactin groups consumed a 60% fat diet and received 30 mg/kg STZ injections twice a week. At an 80 mg/kg body weight dose, surfactin was administered to the surfactin groups, and the detailed schedule is presented in Figure 1A.

In the next experiment, the manufacturer of all experimental mice is the same as above. We redesigned the group to investigate the surfactin's ameliorative mechanism on T2DM mice (prevention or treatment). The feeding environment are the same as described above. Subsequently, Subsequently, the mice were randomly divided into four groups, each make up of eight mice.. The control group and T2DM group were similar to the first experiment. Specifically, the surfactin prevention group (surfactin-p) was supplemented surfactin (80 mg/kg of body weight) after acclimatizing for seven days, and the surfactin treatment group (surfactin-t) was supplemented surfactin (80 mg/kg of body weight) after injecting STZ. The detailed experimental procedures are shown in Figure 7A. Compositions and nutritional ingredients of the experimental diets were shown in the Table S2.

Prior to STZ induction, the mice were fasted for 12 h, after which their FBG levels were measured. STZ was administered twice intraperitoneally at 30 mg/kg body weight to the T2DM and surfactin treatment groups every week, respectively^[17]. In mice that had received STZ injections for three days, the FBG was measured using Roche's blood glucose tester with strip

(ACCU-CHEK@ Performa, Germany) at their tail tip. Mouse models with FBG levels of ≥ 7.8 mmol/L were categorized as T2DM^[17].

2.4 Measurement of body weight, food intake, water intake, and fasting blood glucose

A 7-day food and water intake monitoring program were carried out. The data are exhibited in Figure S1. A Roche blood glucose tester with a strip was used during the experiments to determine FBG every seven days.

2.5 Measurement of oral glucose tolerance

During the final week of the study, we conducted an oral glucose tolerance test (OGTT). Briefly, fasted mice were orally given 1.5 grams of glucose per kilogram of body weight after 12 hours of fasting. After glucose loading, a Roche blood glucose device with a strip (ACCU-CHEK @ Performa, Germany) was employed to detect blood glucose at 0, 30, 60, 90, and 120 min.

2.6 Measurement of insulin tolerance test

During the final week of the study, we conducted an insulin tolerance test (ITT). Briefly, fasted mice were intraperitoneally given 0.75 U of insulin per kilogram of body weight after 12 hours of fasting. After insulin loading, blood glucose at 0, 30, 60, 90, and 120 min was tested using the device mentioned in 2.5.

2.7 Collection and preparation of samples

Avertin-1.25% mice anesthetic, a dose of 0.2 ml/10 g body weight, was used to sacrifice them humanely at the end of the trial to gather their liver and skeletal muscle and adipose tissue. Histological examination of part of the tissues was performed in 4% neutral formaldehyde solution, while the remaining tissue was stored at -80 °C for further research. Commercial kits were used within two weeks to measure the physicochemical indexes of serum.

2.8 Histological analysis

Mice's adipose and liver tissues were excised and fixed in neutral formalin 4%, followed by dehydration and paraffin treatment to evaluate microscopic changes. Haematoxylin & eosin (H&E) and oil red (O-red) staining were performed on paraffin-embedded formalin-fixed liver and adipose tissue blocks sliced into 4 mm thickness.

2.9 Dosage Information

According to our research, surfactin (40, 80, and 160 mg/kg of body weight, oral) protected against T2DM mice. And the results suggested that 80 and 160 mg/kg of surfactin administration possessed a better effect on improving biochemical indexes of T2DM mice. Due to the cost, we thus chose 80 mg/kg of body weight surfactin by gavage every day, to further investigating the detailed mechanism of surfactin against T2DM mice, in the current study. In addition, the results and detailed discussion for dosage Information were shown in the supplementary files (Table S1 and Figure S2-6).

2.10 Western blot analysis

An extraction buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) was used to extract whole proteins. We performed western blotting on the supernatant of whole-cell lysates by centrifuging at 14000g for 5 minutes at 4°C. Separation of proteins was carried out using a one-step PAGE gel fast preparation kit (12%) and protein markers, and the protein was then transferred to the NC membrane. TBST supplemented with 5% skim milk and Tris-buffered saline were used to block membranes for 2 h, followed by overnight incubation at 4°C with diluted primary rabbit antibodies. TBST solution was used to wash the membrane three times, followed by 2 hours of incubation with a secondary antibody. A final scan of the bands was performed using

the ECL plus solution. ImageJ was used to quantify target band density. In addition, as an internal standard, GAPDH was used.

2.11 Statistical analysis

Data were analyzed using SPSS 26.0 program (SPSS Inc., Chicago, IL, USA). The data are shown as the means $\pm$ standard deviations (SD). With SPSS statistics, one-way analysis of variance (ANOVA) was used to determine differences between the groups, followed by Duncan's test. $P < 0.05$ indicated significant differences. In addition, GAPDH is used to normalize the levels of target protein, the detailed methodology is presented in a supplementary file.

3. Results

3.1 Effect of surfactin on critical proteins associated with glucose metabolism in the liver, skeletal muscle and adipose tissue

Our previous study demonstrated that dietary supplementation with surfactin in mice can alleviate HFD/STZ-induced T2DM by regulating intestinal microbiota. To further examine the regulatory mechanism of surfactin on glucose metabolism *in vivo*, we analysed key proteins associated with glucose metabolism in mice with T2DM induced by HFD/STZ. The critical proteins associated with the insulin signalling pathway, glycogen synthesis and gluconeogenesis were analysed in livers using Western blotting. HFD/STZ treatment significantly inhibited hepatic glycogen synthesis, while surfactin administration significantly promoted hepatic glycogen synthesis (Figure 1C). In the liver, PI3K protein levels were dramatically upregulated as a result of HFD/STZ treatment, whereas surfactin dramatically downregulated of T2DM ($p < 0.05$; Figure 1D). Interestingly, surfactin treatment noticeably increased PI3K phosphorylation at the Tyr607 site and the ratio of p-PI3K (Tyr607) to PI3K (p-PI3K/PI3K), as compared with those of the

T2DM mice ($p < 0.05$; Figure 1E). Surfactin noticeably downregulated Akt protein levels, but noticeably upregulated protein levels of Akt phosphorylation at the Ser473 site, as well as the ratio of p-Akt (Ser473) to Akt (p-Akt/Akt), as compared with those of the T2DM mice ($p < 0.05$; Figure 1F-G). Furthermore, the AMPK signalling pathway is also activated by surfactin, which ameliorates hepatic glucose metabolism, according our previous report^[16]. According to these findings, surfactin causes glucose metabolism disorders to be alleviated through the stimulation of hepatic AMPK/PI3K/Akt pathway. As a result of surfactin, mice showed lower hepatic protein levels of PEPCK than mice in the T2DM group ($p < 0.05$; Figure 1H). Surfactin did not dramatically affect hepatic protein levels of GSK-3 β , FOXO1 and GS (Figure. 1I-K). In addition, the protein level of GS in T2DM group is lightly higher than that in the surfactin group ($p > 0.05$, Figure 1C), while glycogen in T2DM group significantly decreased compared to the surfactin group ($p < 0.05$, Figure 1C). GS enzymes with some special modifications can regulate and promote glycogen synthesis. According these findings, it is speculated decreased glycogen content may be due to the low GS activity in the T2DM group.

In skeletal muscle, surfactin treatment also significantly upregulated p-PI3K/PI3K, p-Akt/Akt and the protein levels of GS ($p < 0.05$; Figure 2C, and 2I-K). And a significant amount of GLUT4 was translocated to the cell membrane as a result of surfactin ($p < 0.05$; Figure 2C, and 2I-K). Additionally, noticeably decreased protein levels of GSK-3 β were observed after oral surfactin as compared with those of the T2DM mic ($p < 0.05$; Figure 2L). Surfactin also significantly promoted muscle glycogen synthesis compared to the levels of glycogen synthesis in the T2DM mice ($p < 0.05$; Figure 2M). However, the protein levels of FOXO1 were dramatically decreased as a result of surfactin, compared the T2DM mice ($p < 0.05$; Figure 2H). The protein levels of Akt,

AMPK, p-Akt/Akt and the ratio of p-AMPK (Thr172) to AMPK (p-AMPK/AMPK) were not altered as a result of surfactin ($p > 0.05$; Figure 2D-G). Surfactin showed different changes in GSK-3 β , FOXO1 and GS in liver and skeletal muscle (Figure 1-2), this may be due to STZ/HFD-stimulated different effects on liver and skeletal muscle in T2DM mice.

In adipose tissue, surfactin treatment resulted in higher protein levels of Akt, PI3K, and p-PI3K/PI3K and GLUT4 in the membrane of T2DM mice ($p < 0.05$; Figure 3B, D-F). Nevertheless, p-Akt/Akt and GLUT4 translocation in mice with T2DM were not altered as a result of surfactin ($p > 0.05$; Figure 3C, and 3G).

3.2 Effect of surfactin on critical proteins associated with the inflammatory response in livers, skeletal muscle and adipose tissue

Considering that metabolic inflammation contributes to the development of IR, critical proteins associated with inflammatory cascades in liver, skeletal muscle and adipose tissue were analysed. In the liver, surfactin noticeably suppressed the release of TNF- α , IL-6, IL-1 β and the protein levels of ASC ($p < 0.05$; Figure 4A-D and 4G), while NF- κ B was not significantly different after treatment with surfactin as compared with those of the T2DM group ($p > 0.05$; Figure 4H). Furthermore, HFD/STZ treatment increased the protein levels of JNK and ERK compared with the control mice. However, T2DM mice exhibited lower protein levels of JNK and ERK after surfactin supplementation ($p < 0.05$; Figures 4E-F). The protein levels of p-NF- κ B and the ratio of p-NF- κ B to NF- κ B (p-NF- κ B/NF- κ B) of the T2DM mice were significantly increased as compared with those of normal mice, while surfactin dramatically reduced these parameters compared to the T2DM mice ($p < 0.05$; Figures 4I-J). The protein level of NLRP3 and TLR4 were not significantly altered in the liver in T2DM mice after surfactin supplementation ($p > 0.05$;

Figures S7 A-C). In addition, HFD/STZ dramatically increased protein levels of p-JNK and p-ERK, as well as p-ERK/ERK and p-ERK/ERK, compared to normal mice. These parameters were dramatically decreased by surfactin compared to the mice with T2DM ($p < 0.05$; Figures 4K-M and Figure S7).

In skeletal muscle, HFD/STZ treatment also increased the protein levels of JNK and ERK compared to normal mice ($p < 0.05$; Figure 5A-C). However, surfactin-treated T2DM mice, had significantly lower proteins levels of JNK and ERK e ($p < 0.05$; Figure 5A-C). The levels of NF- κ B, p-NF- κ B, p-NF- κ B/NF- κ B, TNF- α and IL-6 in the T2DM mice were significantly higher compared to those of normal mice, while a significant reduction of these cytokines was observed following surfactin treatment ($p < 0.05$; Figure 5D-H). Additionally, IL-1 β levels were not altered as a result of surfactin compared to those of T2DM mice ($p > 0.05$; Figure S7 H-I). Surfactin supplementation also significantly lowered p-JNK/JNK, p-ERK/ERK, p-JNK and ERK in T2DM mice ($p < 0.05$; Figure 5 I-K and Figure S7).

In adipose tissue, HFD/STZ-induced mice exhibited higher protein levels of JNK and ERK after surfactin treatment ($p < 0.05$; Figure 5L-N), although their protein levels were similar to those of normal mice. When compared with the T2DM group, the levels of IL-6, TNF- α , p-JNK/JNK, p-ERK/ERK, p-JNK and p-ERK were not significantly altered as a result of surfactin as compared with those of the T2DM mice ($p > 0.05$; Figure 5O-Q and Figure S7 N-T).

3.3 Effects of surfactin on histopathology

Liver sections of normal mice (Figure 6A-B) showed normal hepatic architecture with centrally located nuclei, according to H&E and Oil red staining analysis. Liver sections of HFD/STZ-induced T2DM (Figure 6A-B) showed macrovesicular steatosis and vacuolation. Liver

sections of surfactin-treated mice showed hepatocytes with few microvesicular steatosis and vacuolation, this is similar to normal hepatic architecture (Figure 6A-B). In addition, damaged adipocytes were found in HFD/STZ-treated mice, while surfactin treatment significantly repaired damaged adipocytes (Figure 6C-D). HFD caused adipocyte hypertrophy, and subsequent treatment with surfactin resulted in less hypertrophy (Figure 6C-D), as seen by H&E and Oil red staining.

3.4 The effect of surfactin on FBG, OGTT and ITT of mice with T2DM

In this section, the detailed experimental procedures and grouping scheme are shown in Figure 7A, the grouping scheme was applied to the following data. Every week, all mice had their FBG levels measured. Figure 7B indicates that the FBG of the T2DM group was dramatically increased than the FBG of normal mice ($p < 0.05$; Figure 7B). The FBG of mice in the surfactin-p group was dramatically decreased than that of the T2DM mice following surfactin supplementation ($p < 0.05$; Figure 7B).. The FBG of the surfactin-t group were not dramatically alter as compared with those of the T2DM mice ($p > 0.05$; Figure 7B). To further examine whether the alleviating effect of surfactin on T2DM is more preventive or treatment, the effectiveness of surfactin in mice with T2DM was assessed using FBG, OGTT and ITT as indicators.

As a result of the OGTT, all mice's blood glucose levels increased within 30 minutes of oral glucose gavage, and the T2DM mice remained high for 60 minutes afterwards (Figure 7C). Furthermore, the OGTT curve of the surfactin-p group almost coincided with that of normal mice. Despite this, there were no obvious differences between the surfactin-t and T2DM groups in terms of blood glucose levels ($p > 0.05$; Figure 7C). The area under the curve (AUC) also were employed to display the results of the OGTT (Figure 7E). The AUC of the T2DM group was substantially upregulated as compared to that of normal mice, while the AUC of the surfactin-p

group was significantly downregulated as compared to that of T2DM mice ($p<0.05$; Figure 7E).

Additionally, the AUC in the surfactin-t group was slightly lower than for the T2DM mice. This result indicates that surfactin supplementation in the early stage is more effective than in the late stage for ameliorating T2DM.

Figure 7D, the blood glucose level of normal mice recovered $62.8\% \pm 0.6$ mmol/L following 120 minutes of insulin injection, while in the T2DM group, it recovered $46.8\% \pm 0.5$, and the blood glucose levels in the surfactin-p and surfactin-t groups recovered $58.3\% \pm 0.7$ and $62.3\% \pm 1.3$, respectively. These data indicate that insulin tolerance was disrupted in mice with T2DM, and surfactin significantly increased insulin tolerance in both the surfactin-p and surfactin-t groups as compared to that of the T2DM mice ($p<0.05$; Figure 7D). The ITT results are also shown as the AUC within 120 min (Figure 7F). The AUC of ITT in the T2DM group (9.2 ± 0.89 mmol/L·h) was noticeably higher than that in the control group (7.12 ± 0.67 mmol/L·h). Compared with the T2DM group, the AUC of ITT in the surfactin-p group (7.5 ± 0.43 mmol/L·h) was significantly lower ($p<0.05$; Figure 7F), while in the surfactin-t group (8.45 ± 0.87 mmol/L·h), it was slightly decreased ($p>0.05$; Figure 7F). This indicates that surfactin can enhance insulin sensitivity in peripheral tissues, further increasing the utilization of glucose and reducing blood glucose. In addition, surfactin supplementation at the early stage was more effective in alleviating T2DM than at the late stage.

4. Discussion

According to the infrared spectroscopy and mass spectrum analysis, the amino acid sequence of surfactin at position 7 was Ile or Leu or Val, and the chain length of fatty acid was C_{13} - C_{15} . Collectively, we obtained the primary structure of surfactin as Figure S9. In our previous study,

surfactin ameliorated IR by stimulating GLUT4 translocation and suppressing the protein level of PEPCCK via stimulating the PI3K/Akt and AMPK signalling pathways and reducing proinflammatory cytokines and oxidative stress *in vitro*. *In vivo*, dietary supplementation with surfactin also alleviated the mice with T2DM induced by HFD/STZ through regulating gut microbiota. Other components of *B. amyloliquefaciens* fmb50 may play a synergistic role in improving insulin resistance in T2DM mice according our study (the data have been not published), and their effects are not discussed in this study. Previous results have indicated that surfactin could not be broken down and maintain initial bioactivity after digesting (oral administration and gastrointestinal digestion)^[16], it is the basis of this study. To help us further understand these effects, we investigated surfactin's effects in T2DM mice, focusing on the organs (including the liver, white adipose tissue and skeletal muscles) that are affected by insulin. Insulin resistance can be caused by several factors, and this study attempted to identify the mechanism underlying this resistance remission as a result of surfactin.

Lots of previous research have revealed that inflammation plays a key role in insulin resistance pathogenesis^[18-30]. Extensive research has suggested that cytokines (TNF- α , IL-6, IL-1, etc.) are activators of intracellular pathways that promote insulin resistance and T2DM^[29, 31, 32]. Here, decreased cytokine IL-6 levels were observed in adipose tissue after treatment with surfactin in mice with HFD/STZ-induced T2DM. In this study, surfactin noticeably inhibited the liver levels of TNF- α , IL-6 and IL-1 β in T2DM mice. These results also confirmed our previous speculation in hepatic cells^[13]. When GLUT4, insulin receptor, and insulin receptor substrate 1 (IRS-1) protein levels in adipose tissue are low in adipose tissue, it can cause insulin resistance to occur. This condition can be reversed by activating downstream PI3K/Akt and downregulating TNF- α ^[33].

Similarly, HFD/STZ significantly upregulated muscle TNF- α and IL-6 release, while surfactin supplementation dramatically downregulated these cytokines secretion in T2DM mice. Interestingly, surfactin did not alter TNF- α protein levels but promoted GLUT4 and p-PI3K protein levels in adipose tissues and activated the insulin signalling pathway. This might be due to downregulated cytokine IL-6 protein levels. TLR4, a pattern-recognition receptor^[34] is capable of activating the NF- κ B pathway and inflammatory cytokine secretion^[35, 36]. Here, HFD/STZ treatment and surfactin administration did not significantly inhibit TLR4 protein levels, but surfactin significantly downregulated p-NF- κ B and p-NF- κ B /NF- κ B in the liver compared to the T2DM mice, indicating that surfactin-mitigated insulin resistance might be due to reduced inflammatory cytokines release by inactivating NF- κ B signalling pathway independent of the activation of TLR4. Our experiments demonstrated that inactivated NF- κ B cannot inactivate NLRP3 inflammasome in the current study. Additionally, the c-Jun N-terminal kinase (JNK), and the NF- κ B pathway, both of which are linked to IR and T2DM are activated by TNF- α ^[25]. Therefore, it is speculated that the inactivated NF- κ B might be attributed to the inactivation of the TNF- α signalling pathway under the influence of surfactin, and inactivated NF- κ B signalling pathways after surfactin supplementation may partially play roles in protective effects in T2DM mice.

As well as impairing insulin action, oxidative stress also inhibits insulin signalling pathways in cells^[24]. The three main mitogen-activated protein kinase (MAPK) subfamilies involved in ROS production are p38, JNK and ERK, which can induce β -cell deterioration and IR through promoting apoptosis and other ways^[37-40]. However, our findings indicate that adipose tissue levels of p-JNK and p-JNK/JNK, p-ERK and p-ERK/ERK were not significantly altered, while

hepatic and skeletal muscle levels of p-JNK, p-JNK/JNK, p-ERK and p-ERK/ERK were dramatically downregulated as a result of surfactin as compared to those of the T2DM mice. In addition, JNK, an upstream target of NF- κ B, and its phosphorylation can enhance the activation of NF- κ B^[24]. Here, deactivated NF- κ B might also suppress JNK phosphorylation under surfactin effects (Figure 4,5 and S7).

Under insulin-stimulated conditions, phosphorylated PI3K activates Akt, and then glucose production in the liver is suppressed by two critical mechanisms: the first effect is decreased expression of progluconeogenic enzymes due to phosphorylation and nuclear exclusion of FOXO1, a forkhead box protein, and its pro-gluconeogenic targets. the second effect is, activated glycogen synthase (GS) due to phosphorylation and inhibition of glycogen synthase kinase-3 β (GSK-3 β)^[25, 41, 42]. AMPK, a phylogenetically conserved intracellular energy sensor, can regulate insulin sensitivity through regulating PI3K in the liver. PEPCK and G6P, critical gluconeogenic rate-limiting enzymes, were upregulated in T2DM mice in our study. However, surfactin administration to insulin-resistant mice potentiates insulin action and augments insulin signalling via PI3K/Akt and inactivates negative insulin signalling regulators. In detail, surfactin significantly upregulated the protein levels of p-PI3K, p-PI3K/PI3K p-Akt/Akt, p-AMPK (previous study)^[16] and hepatic glycogen synthesis and downregulated GSK3 β protein levels in the liver of T2DM mice as compared to those of the T2DM mice. Surfactin dramatically reduced hepatic PEPCK protein levels in mice with T2DM induced by HFD/STZ. This result is consistent with our group's previous research on the effect of surfactin in IR-HepG2 cells^[13]. In the skeletal muscle, surfactin supplementation also stimulated the PI3K/Akt signalling pathway by increasing protein levels of p-PI3K, p-Akt, p-PI3K/PI3K and p-Akt/Akt, and enhanced muscle glycogen

production was also due to upregulated GS protein levels and downregulated GSK-3 β protein levels after surfactin treatment in mice with T2DM induced by HFD/STZ. On the other hand, surfactin dramatically downregulated FOXO1 protein levels in muscle in mice with T2DM, this is consistent with previous reports^[43, 44]. Collectively, these results indicate that surfactin inhibited gluconeogenesis and enhanced both hepatic and muscle glycogen synthesis mainly via activating AMPK and PI3K/Akt signalling pathways.

Insulin mediates glucose metabolism not only via inhibiting hepatic glucose production but also via enhancing glucose uptake by adipose tissue and skeletal muscle^[20]. It is only GLUT4 that facilitates glucose transport into cells while modulating insulin-induced signaling via the PI3K/Akt/GLUT4 pathway^[45]. A decrease in GLUT4 translocation and activity can lead to increased insulin resistance (IR) and hyperglycemia^[46]. Similarly, in skeletal muscle, GLUT4 protein levels was not altered, while GLUT4 translocation was noticeably promoted after surfactin treatment in mice with T2DM induced by HFD/STZ. In the adipose tissue, surfactin dramatically regulated GLUT4 protein levels, but did not significantly alter GLUT4 translocation as compared to those of the T2DM mice. This is not agreement with previous reports^[46-47], this may be owing to significantly upregulated GLUT4 levels in the membrane as a result of surfactin compared to those of the T2DM mice. These suggest that upregulated GLUT4 levels and activated GLUT4 translocation under surfactin intervention also play critical roles in lowering blood glucose. These findings support our previous results in HepG2 cells^[13]. A pathway regulated by AMPK regulates energy metabolism without being dependent on insulin^[45, 48]. Activated AMPK can promote glucose uptake by muscle cells through GLUT4 translocation^[49]. Here, surfactin did not significantly upregulate muscle p-AMPK protein levels compared to that of the T2DM mice. As

well as inhibiting gene expression of PEPCK and G6Pase, liver-activated AMPK prevents other gluconeogenic gene expression^[50]. Similarly, surfactin administration significantly increased hepatic p-AMPK protein levels and p-AMPK/AMPK as compared to those of the T2DM mice, indicating that surfactin activated AMPK signalling pathway in T2DM mice^[16]. In addition, a previous study demonstrated that surfactin regulated AMPK signaling pathway via modulating gut microbiota at gut-liver axis, suggesting that gut may be a direct target of surfactin in T2DM mice, according to obtained evidence^[16]. Collectively, these findings indicate that surfactin ameliorated IR likely due to the enhancement of GLUT4 translocation and glycogen and downregulation of protein levels of PEPCK and G6Pase via stimulation of the PI3K/Akt and AMPK signalling pathways. In addition, the second experiment indicate that early intervention with surfactin has an ameliorating effect on mice with T2DM induced by HFD/STZ.

In conclusion, oral surfactin can ameliorate IR and lower blood glucose by stimulating insulin signalling, promoting adipose and muscle glucose consumption, enhancing muscle and hepatic glycogen synthesis and suppressing hepatic gluconeogenesis through stimulation of PI3K/Akt and AMPK signalling pathways, as well as reducing proinflammatory factors and oxidative stress through the inactivation of TNF- α , NF- κ B and MAPK signalling in mice. In addition, we confirmed that early surfactin intervention possesses a preventive effect on mice with T2DM induced by HFD/STZ.

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FIGURE CAPTIONS

Figure 1. The experimental schedule (A). Effects of surfactin on glucose metabolism of the liver in HFD/STZ-induced T2DM mice. The western blot results (B), glycogen production (C), the protein level of PI3K (D), p-PI3K/PI3K (E), Akt (F), p-Akt/Akt (G), PEPCK (H), GSK-3 β (I), FOXO1 (J), GS (K). The control group consisted of normal mice fed a basic diet, the T2DM group consisted of T2DM mice fed a 60% fat diet and treated with 30 mg/kg body weight STZ, and the surfactin group consisted of T2DM mice treated with 80 mg/kg body weight surfactin. All data are presented as the means $\pm$ SDs for each group. Different lowercase letters over bars indicate statistically significant differences between two groups ($p < 0.05$). Significance of the mean values for each group was determined using ANOVA and Duncan's test.

Figure 2. Effects of surfactin on glucose metabolism of the skeletal muscle in HFD/STZ-induced T2DM mice. The western blot results (A), the protein levels of PI3K (B), p-PI3K/PI3K (C), Akt (D), p-Akt/Akt (E), AMPK (F), p-AMPK/AMPK (G), FOXO1 (H), GLUT4 in membrane (I), GLUT4 cytoplasm/membrane (J), GS (K), GSK-3 β (L) and glycogen production (M). The control group consisted of normal mice fed a basic diet, the T2DM group consisted of T2DM mice fed a 60% fat diet and treated with 30 mg/kg body weight STZ, and the surfactin group consisted of T2DM mice treated with 80 mg/kg body weight surfactin. All data are presented as the means $\pm$ SDs for each group. Different lowercase letters over bars indicate statistically significant differences between two groups ($p < 0.05$). Significance of the mean values for each group was determined using ANOVA and Duncan's test.

Figure 3. Effects of surfactin on glucose metabolism of the adipose tissue in HFD/STZ-induced T2DM mice. The western blot results (A), the protein levels of Akt (B), p-Akt/Akt (C), PI3K (D), p-PI3K/PI3K (E), GLUT4 in membrane (F) and GLUT4 cytoplasm/membrane (G). The control group consisted of normal mice fed a basic diet, the T2DM group consisted of T2DM mice fed a 60% fat diet and treated with 30 mg/kg body weight STZ, and the surfactin group consisted of T2DM mice treated with 80 mg/kg body weight surfactin. All data are presented as the means $\pm$ SDs for each group. Different lowercase letters over bars indicate statistically significant differences between two groups ($p < 0.05$). Significance of the mean values for each group was determined using ANOVA and Duncan's test.

Figure 4. Effects of surfactin on the inflammatory responses of the liver in HFD/STZ-induced T2DM mice. The western blot results (A), the protein levels of TNF- α (B), IL-6 (C), IL-1 β (D), JNK (E), ERK (F), ASC (G), NF- κ B (H), p-NF- κ B (I), p-NF- κ B/NF- κ B (J). The western blot results (K) p-JNK/JNK (L), and p-ERK/ERK (M). The control group consisted of normal mice fed

a basic diet, the T2DM group consisted of T2DM mice fed a 60% fat diet and treated with 30 mg/kg body weight STZ, and the surfactin group consisted of T2DM mice treated with 80 mg/kg body weight surfactin. All data are presented as the means $\pm$ SDs for each group. Different lowercase letters over bars indicate statistically significant differences between two groups ($p < 0.05$). Significance of the mean values for each group was determined using ANOVA and Duncan's test.

Figure 5. Effects of surfactin on the inflammatory responses of the skeletal muscle and the adipose tissue in HFD/STZ-induced T2DM mice. The western blot results of the skeletal muscle (A), the protein levels in the skeletal muscle of JNK (B), ERK (C), NF- κ B (D), p-NF- κ B (E), p-NF- κ B/NF- κ B (F), TNF- α (G), IL-6 (H). The western blot results of the skeletal muscle (I) p-JNK/JNK (J), p-ERK/ERK (K). The western blot results of the adipose tissue (L), the protein levels the adipose tissue of JNK (M), ERK (N), The western blot results of the adipose tissue (O), p-JNK/JNK (P), p-ERK/ERK (Q). The control group consisted of normal mice fed a basic diet, the T2DM group consisted of T2DM mice fed a 60% fat diet and treated with 30 mg/kg body weight STZ, and the surfactin group consisted of T2DM mice treated with 80 mg/kg body weight surfactin. All data are presented as the means $\pm$ SDs for each group. Different lowercase letters over bars indicate statistically significant differences between two groups ($p < 0.05$). Significance of the mean values for each group was determined using ANOVA and Duncan's test.

Figure 6. Effects of surfactin on histopathology of the liver and the adipose tissue in HFD/STZ-induced T2DM mice. HE staining of the liver (A), Oil red staining of the liver (B), HE staining of the adipose tissue (C) and Oil red staining of the adipose tissue (D). The control group consisted of normal mice fed a basic diet, the T2DM group consisted of T2DM mice fed a 60% fat diet and treated with 30 mg/kg body weight STZ, and the surfactin group consisted of T2DM mice treated with 80 mg/kg body weight surfactin.

Figure 7. The experimental schedule in the second experiment (A). Effects of surfactin on the fasting blood glucose (B) and OGTT (C), ITT (D), AUC of OGTT (E) and AUC of ITT (F) in HFD/STZ-induced T2DM mice during the second experiment. The control group consisted of normal mice fed a basic diet, the T2DM group consisted of T2DM mice fed a 60% fat diet and treated with 30 mg/kg body weight STZ, and the surfactin-p group consisted of T2DM mice treated with 80 mg/kg body weight surfactin before STZ injection. The surfactin-t group consisted of T2DM mice treated with 80 mg/kg body weight surfactin after STZ injection. All data are presented as the means $\pm$ SDs for each group. Different lowercase letters over bars indicate statistically significant differences between two groups ($p < 0.05$). Significance of the mean values for each group was determined using ANOVA and Duncan's test.

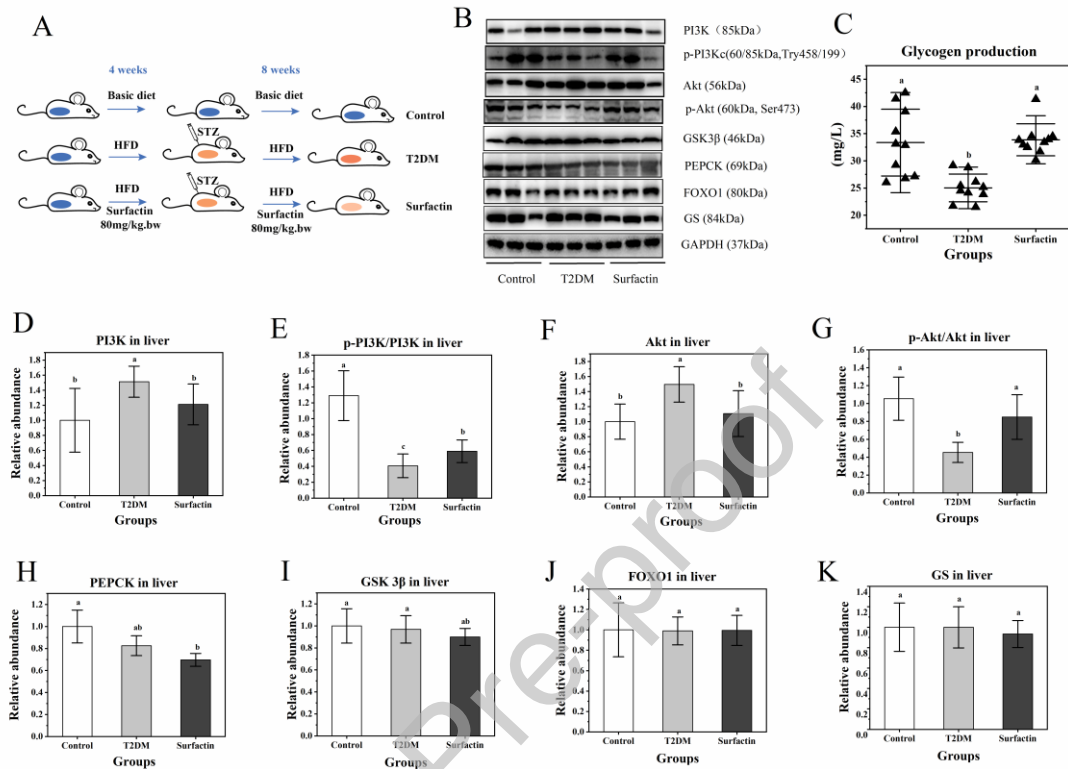
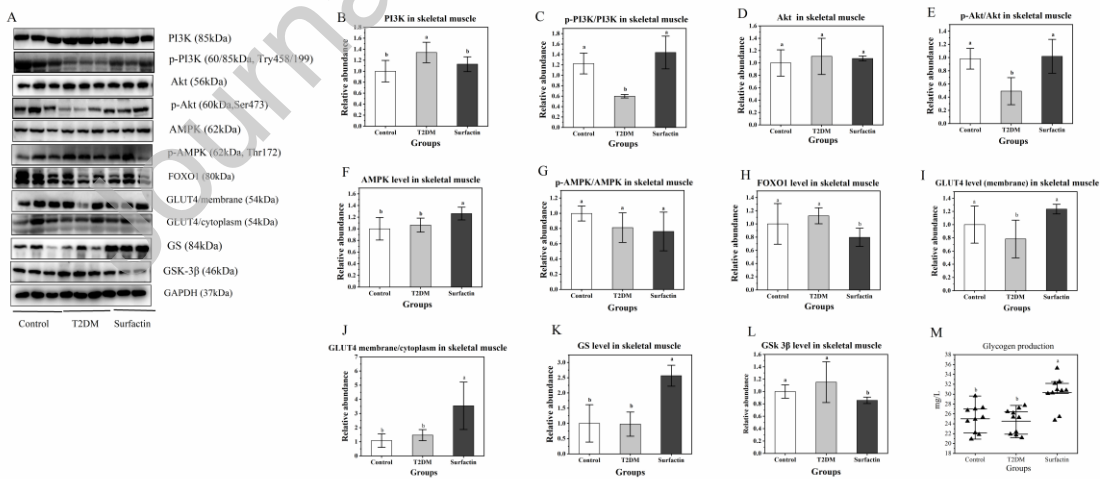
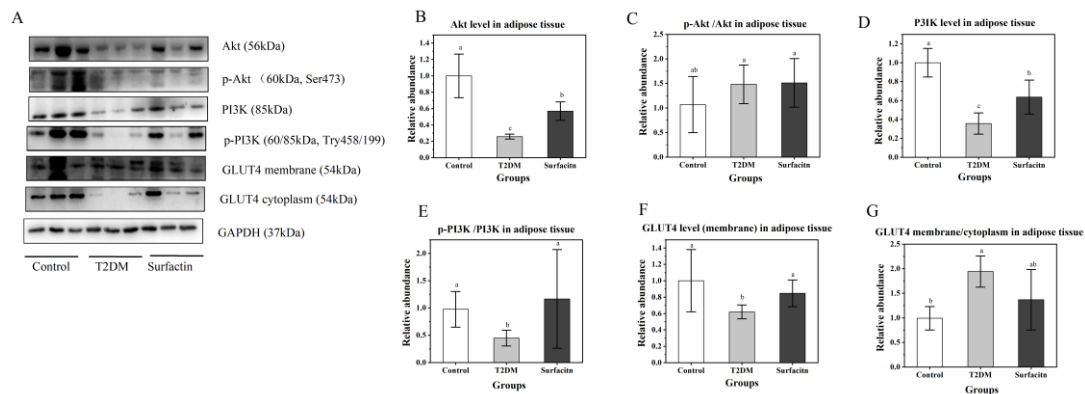
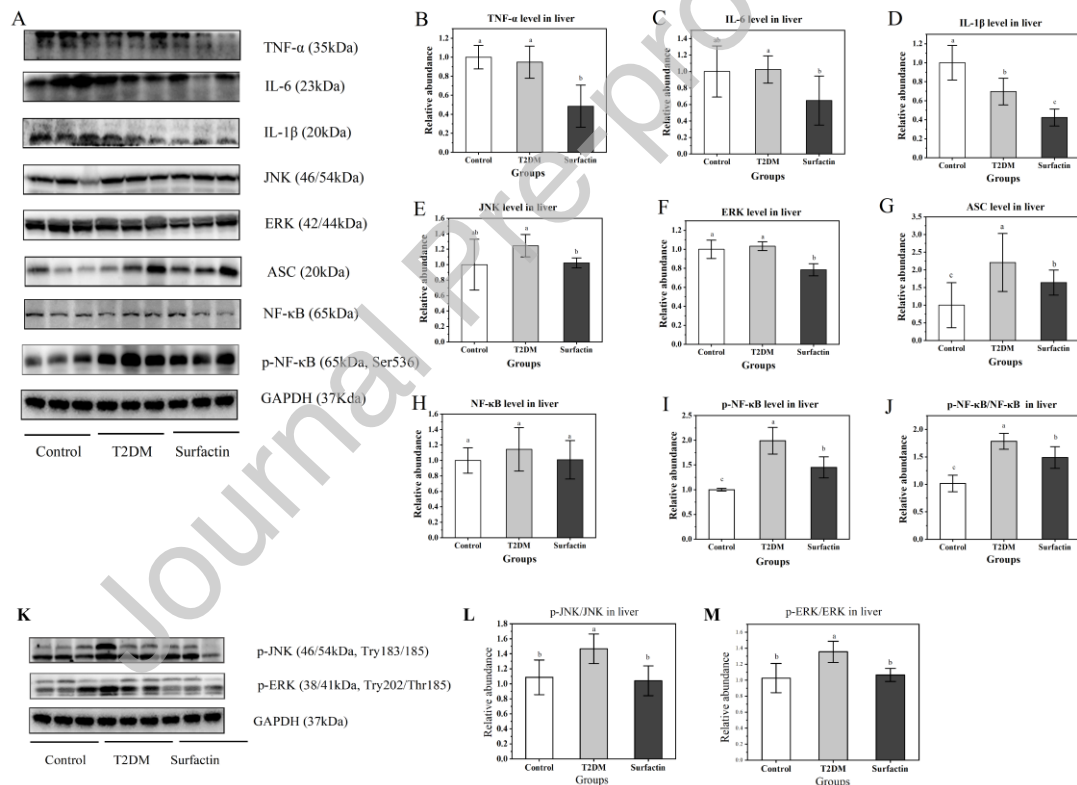
Figure 1.**Figure 2.**

Figure 3.**Figure 4.****Figure 5.**

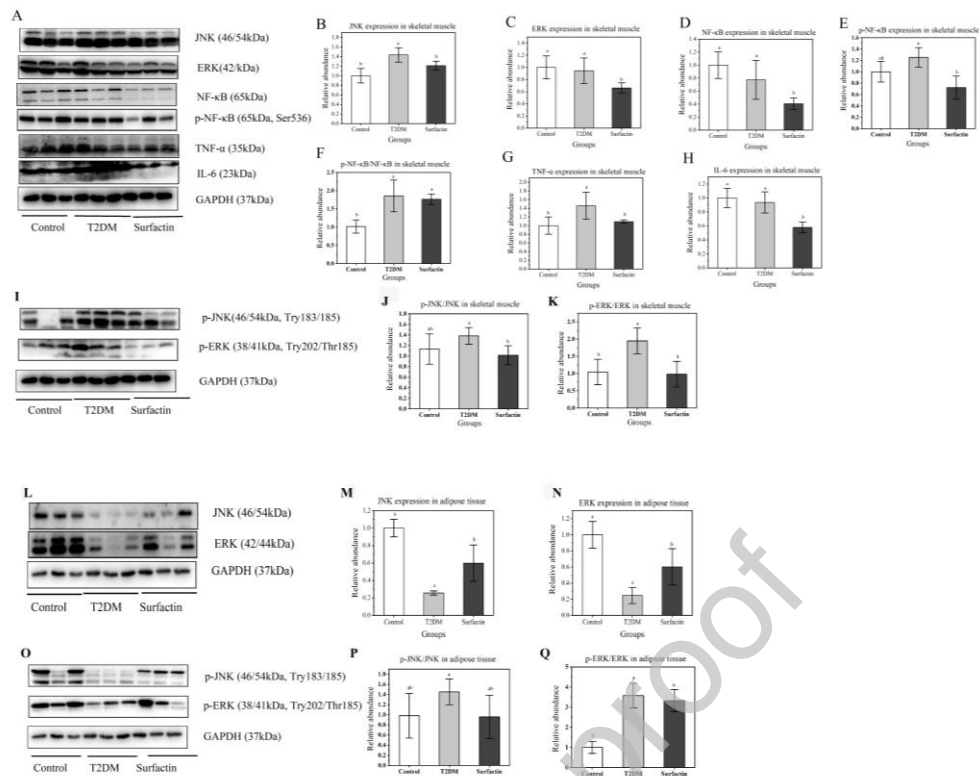


Figure 6.

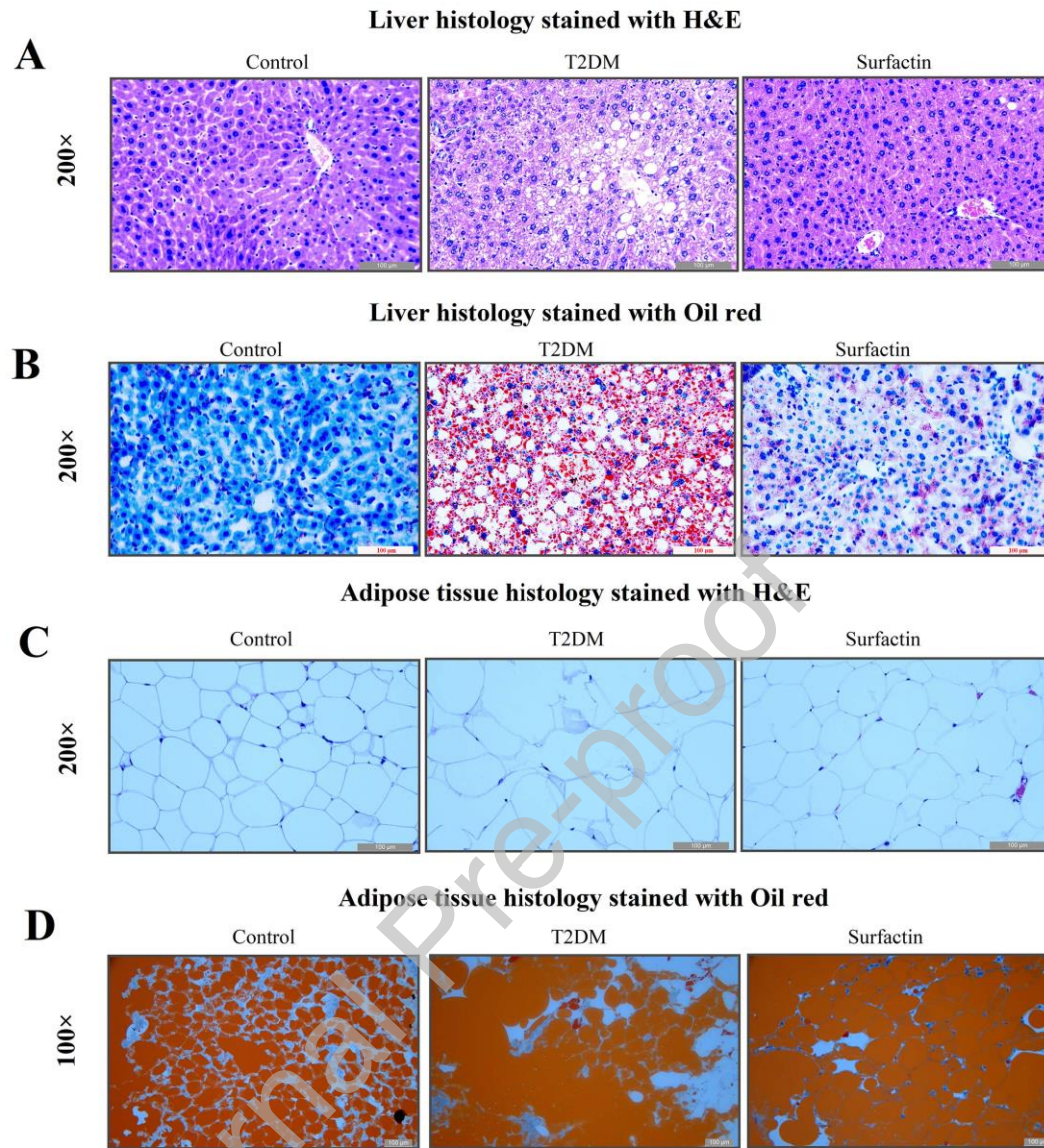
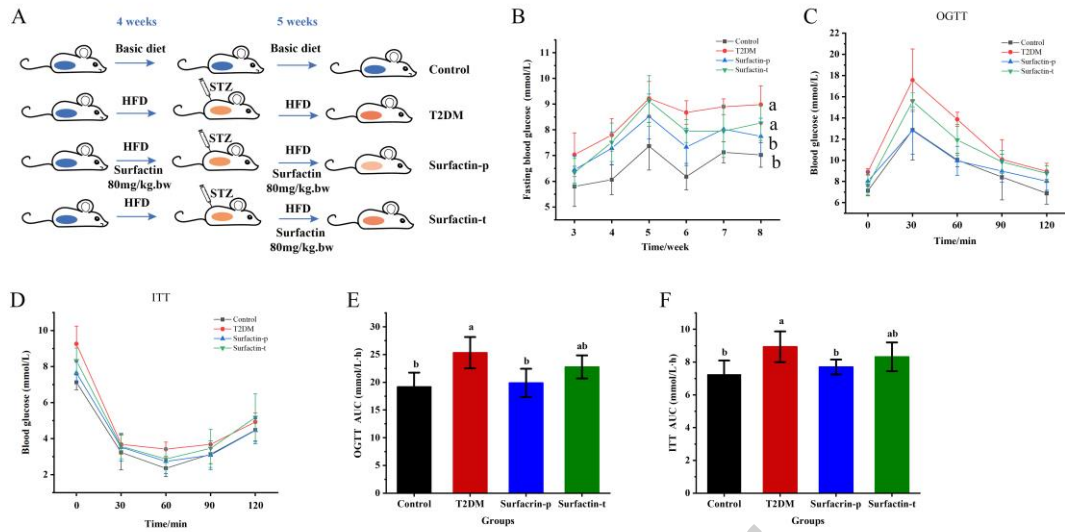
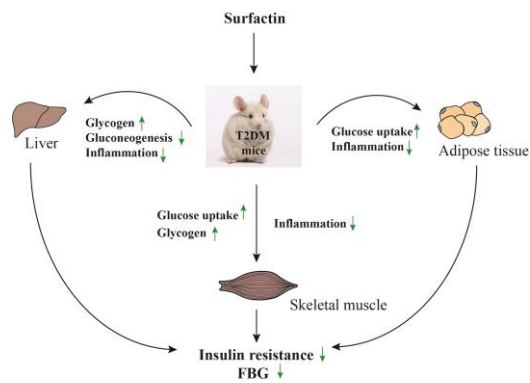


Figure 7.



Graphical abstract.

Potential mechanism by which surfactin alleviates T2DM. Green down arrows indicate decreased parameters or metabolites in the surfactin group, and green up arrows indicate increased parameters or metabolites in the surfactin group.

Conflict of interest

The authors declare that they have no known competing financial interests