1 Metformin alleviates insulin resistance in obese-induced mice via remodeling gut
2 microbiota and intestinal metabolites

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16 Abstract

17 Metformin is widely used to surmount insulin resistance (IR) and diabetes.
 18 Evidence indicates that metformin remodels gut microbiota but the underlying
 19 mechanism remain unclear. Present results showed that metformin effectively
 20 improved insulin sensitivity and alleviated liver inflammation and oxidative stress in
 21 high fat diet (HFD)-induced mice. Metabolomics analysis showed that metformin
 22 increased tauroursodeoxycholic acid (TUDCA) production by increasing the
 23 expression of bile acid synthase *Cyp7a1* and *Baat*. In the palmitic acid (PA)-induced
 24 cell, TUDCA activated Nrf2/ARE pathway, thereby reducing intracellular ROS and
 25 improving insulin signaling. Further gut microbiota analysis showed that metformin
 26 increased the proportion of *Akkermanisia muciniphila* in the HFD-fed mice, while
 27 TUDCA promoted the proliferation of *A. muciniphila* but metformin did not. These
 28 findings reveal that metformin remodels the gut microbiota, reduces oxidative stress
 29 and enhances insulin sensitivity by increasing the production of TUDCA. This
 30 provides a novel mechanism by which metformin alleviates diet-induced IR and
 31 improves metabolism.

32 **Keywords:** Metformin; Gut microbiota; Insulin resistance; Metabolomics; TUDCA;
 33 Nrf2

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Introduction

The prevalence of obesity and overweight have been on the rise worldwide^{1,2}. Current predictions indicate that more than 1 billion people will be obese by 2030³. Visceral obesity precedes the development of metabolic complications and chronic diseases such as type 2 diabetes (T2D)⁴, hepatic steatosis⁵ and cardiovascular diseases⁶. Metformin has been the first-line anti-diabetic drug for more than 60 years because of its excellent hypoglycemic effect and safety profile^{1,7}. Although several studies have explored the mechanisms by which metformin maintains glucose homeostasis, findings from such studies are inconsistent. The prevailing view is that metformin activates AMP-activated protein kinase (AMPK) in the liver, similar to the principle of caloric restriction that relieves symptoms of diabetes^{8,9}. However, metformin is an orally administered drug that reaches high concentrations in the intestine with much lower serum concentrations¹⁰, thus, the possibility that its metabolic therapeutic mechanism might be due in part to actions in the intestine cannot be ignored. Accumulated evidence shows that a high-fat diet (HFD) reduces the abundance of *Lactobacilli* in the small intestine. However, this can be counteracted by metformin, which increases the proportion of probiotics, thus, relieving the symptoms of HFD-induced diabetes¹¹. Although metformin may play a hypoglycemic effect by improving the gut microbiota, oral administration of antibiotics to diabetic mice impairs the hypoglycemic ability of metformin¹². In addition, while metformin can increase the abundance of *Escherichia coli*, it can as well reduce the abundance of *Intestinibacter*. This can further influence diverse biological processes such as the synthesis of branched-chain amino acid and the secretion of GLP-1, thereby improving glucose tolerance and alleviating insulin resistance (IR)^{13,14}. These findings suggest that modulation of gut microbiota is involved in the hypoglycemic mechanism of metformin.

Bile acids (BAs), which are produced in the liver and serve as important ingredients of the digestive fluid in the intestine, enter the small intestine through the

biliary system and participate in fat digestion and absorption^{15,16}. Gathered evidence demonstrates that BAs could improve the intestinal homeostasis by modulating the gut microbiota, thus relieving metabolic syndrome¹⁷. Recent studies also suggest that BAs can participate in the liver-gut axis circulation and the improvement of HFD-initiated metabolic disorders by inhibiting the proliferation of pathogenic bacteria^{18,19}. By modulating the gut microbiota, BAs have also been reported to induce multiple beneficial effects on the intestinal lumen and intestinal walls such as the suppression of intestinal inflammatory responses^{20,21}, the resolution of endoplasmic reticulum (ER) stress in the intestinal epithelial cells underlying the pathology of inflammatory bowel disease²² and the improvement of gut barrier dysfunction²³⁻²⁵. These findings suggest that there is a complex relationship between BAs, gut microbiota and metabolic syndrome.

Previous studies have reported the association of BAs with oxidative stress, which has been shown to treat metabolic dysfunction by acting as an endogenous chemical chaperone to protect cells against ER stress²⁶. Intestinal microenvironment (including inflammation status, the function of the epithelial tight junction and gut microbiota) plays an essential role in the progression of obese-induced IR. The presence of gut microbiota and BAs in the intestine may be closely associated with intestinal metabolic state. Analyses from metagenomic sequencing and metabolomics show that metformin treatment increases the levels of the conjugated bile acid in the gut by decreasing the abundance and bile salt hydrolase (BSH) activity of *Bifidobacterium* species in the intestines of individuals with T2D²⁷⁻³¹. BAs are signal molecules that control the dynamic balance between energy metabolism and liver protection. It has been reported that BAs change with obesity, IR³² and nonalcoholic steatohepatitis (NASH)²⁵. However, the mechanism by which BAs alleviate HFD-induced IR and the underlying role played by the gut microbiota during this process are not clear.

This study investigated how metformin prevents the development of IR in HFD-induced obese mice by modulating the dysbiosis of gut microbiota and alteration metabolites. Exploring the mechanism behind this improves our knowledge of gut microbiota and metabolic interactions underlying the anti-insulin resistance effects of metformin.

Methods

Chemicals and diet

Metformin (purity $\geq 98\%$), choline (purity $\geq 98\%$) and taurine (purity $\geq 99\%$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tauroursodeoxycholic acid (TUDCA, purity $\geq 99\%$) was purchased from Selleck (Houston, Texas, USA). Both the normal chow diet (NCD, containing 10% fat by energy) and high-fat diet (HFD, containing 60% fat by energy) were purchased from Beijing HFK Bio-Technology Co., Ltd. (Beijing, China). Antibodies against Akt, phospho-Akt (Ser473), IRS-1, phospho-IRS-1 (Ser307), AMPK, phospho-AMPK (Thr172), Keap1, I κ B, phospho-I κ B (Ser32), p65, phospho-p65 (Ser536), GAPDH and β -tubulin were obtained from Cell Signaling Technology (Beverly, MA, USA). Nrf2, phospho-Nrf2 (Ser40), and 4-HNE were purchased from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, USA).

Animal experiments

Male ICR mice (7 weeks old, body weight 20 ± 1 g) were housed in ventilated cages (four animals per cage) at the SPF facility of Wenzhou University under controlled environmental conditions (temperature $22 \pm 2^\circ\text{C}$; relative humidity 60–70%) with free access to standard laboratory chow and tap water. The mice were maintained on a regular 12/12 h light/dark cycle. All animal care and experimental procedures were approved by the Animal Care Ministry of Health and were performed in accordance with the guide for the Care and Use of Experimental

Animals of Wenzhou University.

Mice were acclimatized to their environment for 1 week before the experiments. Mice were randomly allocated into three groups (n = 9 for each group). A co-worker blinded to the experimental protocol randomized animals into these groups. One group of animals was fed with NCD and the other two groups were fed with HFD. After 12 weeks of HFD feeding, one group of HFD-fed mice was administered metformin (200 mg/kg body weight), once daily (HFD+Met group) for 6 weeks, whereas the NCD-fed mice (NCD group) and other group of HFD-fed mice (HFD group) were treated with an equal volume of saline for 6 weeks. The whole study lasted 18 weeks, during which the body weight, water consumption and food intake were measured every week. At week 18, intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) were performed as previously described³³.

Fresh feces were collected and stored immediately at 80°C for subsequent analysis. At the end of the trial, after overnight fasting for 12 h, blood samples were collected, and serum was isolated by centrifugation at 1000 × g for 15 min at 4°C, and stored at -80°C for further assay. Tissues, including the adipose tissue, liver and ileum, were weighed; one portion of the tissues was fixed with 10% formaldehyde for histological analysis, and the other portion was immediately frozen in liquid nitrogen for further analysis.

Total cholesterol (T-CHO), triacylglycerol (TG), free fatty acids (FFA), high-density lipoprotein (HDL-c), low-density lipoprotein (LDL-c), fasting blood glucose (FBG), fasting serum insulin (FINS), catalase (CAT), malondialdehyde (MDA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), acid phosphatase (ACP) and alkaline phosphatase (AKP), mice-lipopolysaccharides (LPS) were determined by biochemical kits purchased from Jiancheng Bioengineering Institute (Nanjing, China). The ratio of reduced glutathione (GSH) and oxidized

glutathione (GSSG) were determined by a GSH:GSSG kit (Jiancheng Bioengineering Institute, Nanjing, China). Homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated using the following formula:

$$\text{HOMA-IR} = \text{FBG (mmol/L)} \times \text{FINS (mU)} / 22.5$$

Hematoxylin and eosin staining

Samples were fixed in 10% formaldehyde overnight, paraffin-embedded, sectioned (4- μm thickness, 3–5 sections/specimen) and stained with hematoxylin and eosin (H & E) for histological analysis. Digital images of H & E stained sections were acquired with a Nikon Eclipse Ti microscope at $\times 400$ magnifications (Ti-E/U/S, Japan). Image J software (National Institutes of Health, USA) was used to count adipocytes.

Cell culture

Human liver hepatocellular carcinoma (HepG2) cells were cultured in MEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Gibco, South American), penicillin and streptomycin. Cells were maintained in 5% CO_2 at 37°C .

siRNA Transfection

Nrf2 was knocked-down by RNA interference (RNAi) using the following 19-bp (including a 2-deoxynucleotide overhang) siRNAs (Origene, Beijing, China): *Nrf2*, SR321100A-AUUGAUGUUUCUGAUCUAUCACUTT; SR321100B-GUCAGUAUGUUGAAUCAGUAGUUTC; SR321100C-CCAGUCUUCUUGCUACUAAUCAGG. Stealth RNAi (Origene, Beijing, China) was used as a negative control (siCont). For transfection, cells were seeded on a six-well plate, grown to ~80% confluence and transfected with siRNA duplexes using Lipofectamine 3000 (Invitrogen, Camarillo, CA, USA) according to the manufacturer's recommendations. After incubation for 48 h, the expression level of *Nrf2* protein was detected by western blot.

Glucose uptake assay

Cells were plated at 1×10^4 /well in 96-well plates and used at subconfluence after 24 h of preincubation. For experiments, all culture medium was removed from each well and replaced with 100 μ M fluorescent 2-Deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose (2-NBDG)³⁴ in serum-free medium and incubated for 30 min. Subsequently, cells were washed thrice with PBS and then the fluorescence intensity was determined with a fluorescence microplate reader (Ex/Em, 488/520 nm). The glucose concentration in the medium supernatant was determined by a glucose detection kit (Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

Flow cytometry assays

The reactive oxygen species (ROS) level was evaluated by flow cytometry (FACSCanto II, BD, USA) using the DCFDA cellular reactive oxygen species detection assay kit (Byotime, Shanghai, China) according to the manufacturer's protocol. Cells were briefly stained with 2',7'-dichlorofluorescein diacetate (DCFDA) at 37°C for 30 min, washed with $1 \times$ PBS buffer, and the signal was read at an excitation of 485 nm and an emission of 535 nm.

Quantitative RT-PCR

Total mRNA was isolated from tissue samples using TRIzol reagent (TAKARA, Tokyo, Japan) and was reverse-transcribed into cDNA using a high-capacity cDNA reverse transcription kit (TAKARA, Tokyo, Japan) according to the manufacturer's protocol. The mRNA levels were quantified with quantitative PCR (qPCR) using SYBR Green (Qiagen, Hilden, Germany). Amplification was performed on a LightCycler480 qRT-PCR system (Roche, Mannheim, Germany) under the following reactions: 95°C for 15 min, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. The relative mRNA levels of target genes were normalized to the expression of GAPDH calculated using $2^{-\Delta\Delta Ct}$ method. The primer pairs used in this

study are listed in Supporting table.

Western blot analysis

Samples were homogenized with ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors (Beyotime, Shanghai, China). The homogenates were centrifuged at $10\,000 \times g$ for 20 min at 4°C to remove the insoluble tissue debris. The protein concentration in the supernatant was determined using a BCA protein assay kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Equal amount of protein for each group were denatured in boiling water for 5 min. Aliquots (40 µg) of protein samples were subjected to 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk (dissolved in TBST) for 1 h, the membranes were incubated with the indicated antibodies at 4°C overnight, followed by incubation with the appropriate HRP-conjugated second antibodies for 1 h at room temperature. Chemiluminescent detection was performed using the ECL Plus Western blotting reagent (TransStart, Beijing, China). Semi-quantitative analysis for densitometry of each band was performed using ImageJ software.

Analysis of gut microbiota by 16S rRNA amplicon sequencing

As described previously¹¹, total bacterial DNA was extracted from the collected feces using CTAB/SDS method. Illumina MiSeq (Novogene Bioinformatics Technology Co., Ltd.) was used to analyze the 16S rRNA genes in the DNA sample to determine the composition and diversity of bacteria. Sample processing, data acquisition and analysis were described in full detail in Supplementary methodologies.

Non-targeted metabolome analysis

A non-targeted metabolome analysis of the colon contents was performed with a liquid chromatography-tandem mass spectrometry (LC-MS) method (Waters

ACQUITY UPLC (Waters, America) coupled to a Thermo LTQ Orbitrap XL system (Thermo, America)). Sample processing, data acquisition, UPLC conditions and data analysis were described in full detail in the Supplementary methodologies.

Growth curve of *Akkermansia muciniphila*

Akkermansia muciniphila strain ATCC BAA-835 was purchased from the Beinan (Beijing, China). Bacteria were cultured in BHI medium in tubes at 37°C in an anaerobic chamber (Whitley A35 Workstation 2.5, Don Whitley Scientific, UK). To acquire the growth curve of *A. muciniphila*, the medium in test tubes supplemented with or without 10 mM metformin (or 200 µM TUDCA) was placed in an anaerobic bag to eliminate oxygen for 24 h. The test tubes were then inoculated with bacteria and incubated at 37°C in an anaerobic bag. The OD₆₀₀ of the cultures was measured every 3 hours.

Determination of hepatic TUDCA

The contents of TUDCA in hepatic tissues were determined by high-performance liquid chromatography (HPLC). Briefly, 200 mg of frozen tissue was homogenized with a TissueLyserII (Qiagen, Germantown, MD, USA) in 1 mL of PBS to prepare the tissue homogenates. The impurities were removed through a 0.22 µm filter, then the filtrate was precipitated by methanol and 10 µL of supernatant was analyzed using an Agilent 1290 HPLC system (Santa Clara, CA) equipped with a Hypersil ODS-2 column (5 µm, 4.6 × 250 mm; Waters, USA). The supernatant was detected at the wavelength of 210 nm, eluted with the mobile phase of 0.03 M phosphate buffer solution (pH 4.4) and methanol (32:68, v/v) at a flow rate of 1.0 mL/min.

Statistical analysis

Statistical comparisons among experimental groups were analyzed by one-way ANOVA and Duncan's multiple-comparison test using the SPSS software (Version

21.0. Armonk, NY: IBM Corp.). *P* values less than 0.05 were considered statistically significant.

Results

1. Metformin prevents diet-induced obesity and increases insulin sensitivity

To assess the efficacy of metformin on the treatment of obese mice with IR, animals were divided into the NCD, HFD and HFD + Met groups. Daily oral administration of metformin significantly alleviated diet-induced body weight increase from day 14 onwards (**Figure 1A**). At the end of metformin treatment, the weight gain and BMI were also significantly decreased (**Figure 1B, Supplementary Figure 1a**), without affecting food intake (**Figure 1C**). Metformin prevented fat deposition in the subcutaneous adipose, the adipose index (**Supplementary Figure 1b**) and H & E staining of adipose tissue (**Figure 1D**). Adiposity numbers showed that metformin effectively ameliorated adipocyte hypertrophy (**Figure 1E**). The indexes of the pancreas and kidney were not affected by metformin administration. The liver index was significantly reduced in metformin-treated mice (**Supplementary Figure 1b**). The diet-induced hepatic steatosis and dyslipidemia were effectively prevented by metformin treatment as indicated by the lower concentration of T-CHO, TG and FFA in both the liver and the serum of the HFD + Met group compared with the HFD group (**Figure 1 F-H**). We also found that metformin could effectively prevent hepatocyte damage. This was evidenced by the lower levels of AST, ALT, ACP and AKP in the serum (**Supplementary Figure 2**).

Glycemic homeostasis was determined by IPGTT and IPITT. As shown in **Figure 2A**, the oral administration of glucose increased the blood glucose (BG) level within 30 min in the HFD-fed mice, which remained high for the subsequent 90 min. This indicated that HFD feeding markedly impaired glucose tolerance. In addition, the BG of the NCD group peaked within 30 min but dropped to the normal level after 120 min. Metformin significantly inhibited the rise of BG level and enhanced glucose

clearance in the HFD-fed mice after oral administration of glucose. The results of IPGTT were expressed as the area under the curve (AUC) of BG over 120 min. The AUC of the HFD + Met group was significantly decreased compared with that of the HFD group (**Figure 2B**, $P < 0.05$). The improvement of insulin resistance (IR) was also supported by the results of IPITT. After intraperitoneally injected insulin at 0.75 U/kg, the BG levels at 30, 60, and 120 min revealed that the mice in the HFD + Met group took more advantage of insulin compared with the mice in the HFD group (**Figure 2C**). The significant difference ($P < 0.05$) of the slope between the HFD group and the HFD + Met group suggested that metformin increased the utilization of insulin and improved insulin sensitivity in the HFD-fed mice (**Figure 2D**). Moreover, the FBG level in the HFD group was significantly higher compared to that in the NCD group. Compared with the HFD group, the mice treated with metformin had a lower FBG level (**Figures 2E**). Marked elevation in fasting serum insulin (FINS) was observed in the HFD group compared with the NCD group. Notably, metformin treatment tended to reverse the increase in FINS (**Figure 2F**). HOMA-IR is an indicator for evaluating the level of insulin resistance in an individual. Based on the HOMA-IR analysis, we found that the HOMA-IR of the HFD + Met group was comparable to that of the NCD group but significantly lower compared with that of the HFD group (**Figure 2G**).

PI3K/Akt and insulin receptor substrate-1 (IRS-1) are the main signaling molecules in the signaling pathway of the insulin receptor. The level of phosphorylated Akt at Ser473 was significantly reduced in the liver of the HFD-induced obese mice, but the level was restored in the HFD + Met group (**Figure 2H**). Contrary to the phosphorylated Akt, the level of phosphorylated IRS-1 at Ser307 was significantly elevated in the HFD group, whereas in the HFD + Met group, the level was reduced. These results indicate that the preventive effect of metformin on HFD-induced obesity resulted in improved glucose homeostasis and insulin sensitivity.

2. Metformin alleviates HFD-induced inflammation and oxidative stress

As shown in **Figure 3A** and **3B**, metformin prevented metabolic inflammation in the liver and ileum, leading to an increased tendency towards reduced gene expression of interleukin-1 β (*Il-1 β*), interleukin-6 (*Il-6*) and tumor necrosis factor- α (*Tnf- α*) in the ileum. However, there was no effect on interleukin-10 (*Il-10*). Metformin also prevented the release of endotoxin, which was indicated by lower levels of LPS in serum (**Figure 3C**). Meanwhile, the phosphorylated levels of p65 and I κ B were decreased in the livers of metformin-treated mice compared with the HFD-fed mice (**Figure 3D**). This indicated that the HFD-induced activation of NF- κ B signaling was suppressed by metformin.

To investigate the involvement of oxidative stress in the development of HFD-induced IR, we first measured the activity of the antioxidant enzyme in both the plasma and the liver. The results showed that the activities of CAT in the serum and the liver were markedly lower in the HFD group compared with the NCD group (**Figure 4A**). In addition, the ratio of GSH/GSSG was reduced (**Figure 4C**). After metformin intervention, the activities of CAT and the ratio of GSH/GSSG were significantly increased. The accumulation of lipid peroxide, caused by HFD, was improved in the metformin group (**Figure 4B**). This was exhibited by the significantly reduced MDA level in the liver. Nrf2/ARE is a classic antioxidant-signaling pathway and KEAP1 is a key protein that mediates the degradation of Nrf2. HFD feeding significantly inhibited Nrf2/ARE signaling (**Figure 4D**) whereas metformin significantly increased the expression of Nrf2. Nrf2/ARE signaling regulates the expression of a variety of detoxification and antioxidant enzymes. As shown in **Figure 4E**, Metformin significantly up-regulated the mRNA levels of *Nqo1* and *Hol*, which are essential genes for balancing oxidative stress. These results indicate that metformin can improve intestinal and hepatic inflammation, oxidative stress and lipid peroxide accumulation in HFD-fed mice.

3. Metformin prevents obesity-driven dysbiosis of gut microbiota and reshapes intestinal metabolites

Faecal samples were collected for gut microbiota analysis by 16S rRNA amplicon sequencing. Following all quality trimming and checking, a dataset consisting of 80004, highly qualified reads, was collected for subsequent analysis. Our data showed that the numbers of unique OTUs in the NCD, HFD, and HFD + Met groups were 372, 350 and 374, respectively. The coverage of sequencing results was nearly complete for all sequences in the three groups, demonstrating sufficient sequencing depth for follow-up analyses. The metformin-treated group significantly increased the Ace and Chao1 indices compared with the HFD group (**Table 1**). Metformin-treated mice, however, clustered partially apart from HFD-fed mice samples, suggesting important changes in metformin gut microbial profile (**Figure 5A**). Metformin treatment prevented HFD-induced decrease in Bacteroidetes and the increase in the Firmicutes/Bacteroidetes ratio (**Figure 5B and C**), two hallmarks of obesity-driven dysbiosis. Based on FunGuild and FAPROTAX analysis of flora function, the HFD group showed a decrease in the proportion of the chemoheterotrophy and the fermentation bacteria when compared with the NCD group (**Figure 5D**). However, after metformin supplementation reshaped the structure of gut microbiota, chemoheterotrophy and fermentation improved in the HFD + Met group. Heatmap presents the clustering of bacterial communities with their relative abundances at the genus level (**Figure 6A**). Compared with the NCD group, unique high-abundance bacteria in the HFD group are mainly concentrated in *Bifidobacterium*, *Roseburia*, *Lachnoclostridium*, *Alloprevotella* and *Mucispirillum*. However, metformin decreased the abundance of these bacteria, making the metformin-treated group comparable to the NCD group. In addition, metformin specifically increased the abundance of *Butyricimonas*, *Parasulterella*, *Parabacteroides* and *Akkermansia*. Consistent with the decrease in F/B ratio as shown in **Figure 5C**, the difference between the HFD group and the HFD + Met group is

mainly reflected in the decrease in the abundance of Firmicutes and the increase in the abundance of Bacteroidetes (**Figure 6B**). Compared with the NCD and HFD + Met groups, *c_Erysipelotrichia*, *f_proteobacteria*, *g_Turicibacter*, *o_Bifidobacteriales* and *s_Clostridium* in the HFD group were significantly up-regulated, whereas *f_Muribaculaceae*, *g_parabacteroides* and *f_tannerellaceae* were significantly up-regulated in the HFD + Met group. Therefore, metformin can increase the abundance of Bacteroidia at the phylum level and *Parabacteroides* at the genus level, and modulate HFD-induced dysbiosis of gut microbiota.

The intestinal metabolite profiles were further measured using lipid chromatography and mass spectrometry (LC-MS) to reveal the influences of metformin on the intestinal metabolites. When comparing the HFD group and HFD + Met group, metabolites were dictated by metformin. For instance, in the HFD group, 118 up-regulated metabolites were identified, whereas 182 down-regulated metabolites were established in the HFD + Met group (**Figure 7A-C**). The HFD + Met group effectively increases the content of taurine, choline and TUDCA in the intestine compared with the HFD group (**Figure 7D**). This is consistent with previous reports¹⁸. Collectively, these results showed that metformin administration altered the intestinal metabolites and the gut microbiota. **Figures 8A and 8B** showed the correlation between intestinal metabolism and the gut microbiome at the phylum and species level after the intervention of metformin in HFD-fed mice. TUDCA was highly associated with *A. muciniphila* species belonging to the *Verrucomicrobia* phylum. Interestingly, through the analysis of growth curves, it was not metformin but TUDCA significantly accelerated the proliferation of *A. muciniphila* *in vitro* (**Figure 8C**). These data suggest that metformin may indirectly improve insulin resistance caused by a high-fat diet through modulation of the structure of gut microbiota and metabolites.

4. TUDCA improves PA-induced insulin resistance and lipid peroxidation

Further study was carried out to determine whether TUDCA could alleviate hepatic insulin resistance using a PA-treated HepG2 cell model. PA exposure (200 μ M) resulted in a decrease in glucose uptake in HepG2 cells. This was demonstrated by the reduction of fluorescence density in the cytoplasm (**Figure 9A**) and the high concentration of glucose in the medium (**Figure 9B**). In addition, PA decreased the level of phosphorylated Akt at Ser473 and increased phosphorylated IRS-1 level at Ser307, thus, revealing a typical symptoms of insulin resistance (**Supplementary Figure 3**). As shown in **Figure 9A** and **9B**, the impaired glucose uptake caused by PA was significantly alleviated by TUDCA, excluding choline and taurine (**Supplementary Figure 4**). Moreover, the suppressed phosphorylation level of Akt in PA-treated HepG2 cells was elevated by TUDCA, while the phosphorylated IRS-1 level at Ser307 was also significantly reduced (**Figure 9C**). Note that, TUDCA treatment at high doses may produce toxic side effects and fail to exert insulin sensitization. These findings confirmed that TUDCA could improve hepatic glucose intolerance and enhance insulin sensitivity of PA-treated hepatocyte in a metformin-independent manner.

Homeostasis imbalances on glucose metabolism often cause inflammation and oxidative stress damage in hepatic cells. Thus, we further investigated whether TUDCA could alleviate these pathological processes. The intracellular ROS content was visualized by using the DCF-DA dye and flow cytometric platform. **Figure 10A** showed that PA could increase ROS generation in the HepG2 cells. Interestingly, TUDCA significantly decreased PA-induced ROS generation. On the other hand, the ratio of GSH/GSSG was significantly increased by TUDCA (**Figure 10B**). In addition, pro-inflammatory cytokines such as *Tnf- α* , *Il-1 β* and *Il-6* were also significantly reduced by TUDCA treatment at mRNA level (**Supplementary Figure 5**).

We further investigated whether TUDCA alleviates PA-induced insulin resistance through the Nrf2/ARE signaling pathway. The transcriptional activity of Nrf2 was determined by measuring the mRNA levels of its representative downstream

target genes. TUDCA significantly up-regulated the expression of Nrf2, suppressed by PA (**Figure 10D**). It also up-regulated the expression of antioxidant genes, *Ho-1*, *Nqo1*, *Cat* and *Gpx4*, downstream of the Nrf2/ARE signaling pathway (**Figure 10D**). We found that TUDCA was unable to alleviate PA-induced insulin resistance in HepG2 cells that interfered with Nrf2 expression (**Supplementary Figure 6, 10E & F**). Together, the results suggest that TUDCA may significantly activate the Nrf2/ARE signaling, thereby enhancing antioxidant capacity, and alleviating insulin resistance in the HepG2 cells caused by PA.

To further determine the source of TUDCA, we detected the TUDCA content in the liver of the HFD and the HFD + Met groups. As shown in **Figure 11A**, TUDCA in the liver of the HFD + Met group (5.281 ± 0.745 nmol/g) was significantly increased compared with that in the HFD group (2.992 ± 0.691 nmol/g). In the HFD + Met group, the gene expression of *Cyp7a1* and *Baat*, the important enzymes involved in the biosynthesis of TUDCA, was significantly up-regulated compared with that in the HFD group (**Figure 11B**). This suggests that metformin can promote the production of TUDCA in the liver by up-regulating the enzymes that limit the rate of TUDCA biosynthesis, and *Cyp7a1* and *Baat* expression.

Discussion

Metabolic syndromes such as obesity, nonalcoholic fatty liver disease and T2D are increasingly prevalent globally, especially in recent decades². Metformin, the most widely used anti-diabetic drug worldwide, has been reported to favorably influence metabolic and cellular processes. Metformin is also shown to be closely associated with the development of insulin resistance^{35,36}; however, the mechanism is still not clear. Recent studies have revealed that the development of metabolic disease strongly correlates to the accumulation of gut microbiota and metabolites. Multiple studies have investigated the impact of metformin on the gut microbiota, metabolism and related host targets on the regulation of glucose metabolism^{37,38}. We explored the

potential mechanism involving the gut microbiota and intestinal metabolites to reveal how metformin improves diet-induced obesity and insulin resistance. We established that the daily administration of metformin strongly ameliorated the obesity-related interruption of gut microbiota. The bile acid TUDCA was maintained at a high level in the intestine, which in turn promoted the proliferation of *A. muciniphila*, improved the integrity of the intestinal wall and reduced intestinal inflammation. Furthermore, TUDCA was identified as an activator of Nrf2/ARE signaling in hepatocyte that decreased oxidative stress imbalance, while improving hepatic inflammation and insulin resistance in HFD-induced obese mice.

T2D is both an inflammatory and a natural immune disease. There is a variety of microbiota in the human intestine, which is involved in body immunity, infection and nutrient metabolism, and they are closely associated with the occurrence of diabetes. The gut microbiota imbalance in composition and diversity can result in the release of bacterial endotoxin. This could lead to chronic inflammation and destruction of the normal physiological functions of tissues and organs. Several studies have confirmed that chronic inflammation can cause insulin resistance and subsequently diabetes. The decrease in Firmicutes/Bacteroidetes ratio is considered to be an indicator of improved metabolism by metformin therapy^{39,40}. LDA analysis showed that the decreased abundance of Firmicutes by metformin could be due to the decrease in *Erysipelotrichia* species, which belongs to the Firmicutes phylum. The role of *Erysipelotrichia* species in human disease may be well established in studies investigating metabolic disorders⁴¹. For instance, a previous study showed a bloom of species belonging to the *Erysipelotrichaceae* family (initially classified as *Mollicutes*) in diet-induced obese animals⁴². Within the host, *Erysipelotrichia* species appear to be highly immunogenic⁴³, however, in surplus, they induce intestinal inflammation. Studies have shown that species in *Erysipelotrichaceae* are positively correlated with TNF α and IL-6 in the intestine and the liver⁴⁴.

In contrast, most of the bacteria up-regulated by metformin were probiotics. For example, species in *Muribacaceae* are involved in the L-citrulline nitrogen metabolic cycle. They scavenge free radicals, improve immunity and maintain blood glucose. Metformin also up-regulates the abundance of *Parabacteroides*, including *P. distasonis* and *P. goldsteinii*. Liu et al.⁴⁵ found that *P. distasonis* could transform bile acids, activate intestinal gluconeogenesis through succinic acid and improve the host metabolic disorders. In another study, Wu et al.⁴⁶ recorded that *P. goldsteinii* reduces systemic inflammation and increases insulin sensitivity by maintaining intestinal integrity. *Akkermansia muciniphila* is known to effectively alleviate intestinal inflammation caused by obesity. Patients with intestinal obesity have been shown to have a significantly reduced abundance of *A. muciniphila* compared with healthy individuals. Numerous studies have demonstrated that metformin could up-regulate the abundance of *A. muciniphila* in HFD-fed mice intestine, maintain the integrity of the intestinal wall as well as effectively improve intestinal inflammation⁴⁷. The abundance of *A. muciniphila* is also significantly upregulated in the HFD + Met group. As a mucin-degrading bacterium, the use of *A. muciniphila* has been shown to reduce inflammation, maintain the intestinal barrier and increase levels of endocannabinoids secreted by intestinal peptides⁴⁸. We speculate that the transformation of gut microbiota by metformin is the defining factor in improving chronic inflammation in the intestine and even the whole body. Metformin can effectively inhibit the number of pathogenic bacteria and promote the proliferation of probiotics. A suppression of pathogenic bacteria coupled with a proliferation of probiotics may synergistically reduce intestinal inflammation and maintain the integrity and permeability of the intestinal wall. Furthermore, this may lead to a decrease in serum LPS and improvement of systemic inflammation caused by HFD-induced obesity.

The "dialogue" between the gut microbiota and the organism mainly depends on microbial metabolites. By producing various enzymes for biochemical metabolic pathways of the intestinal microbes, gut microbiota can perform diverse metabolic

activities such as the metabolism of amino acids, carbohydrates and bile acids, as well as the formation of a co-metabolic relationship with the host. Bile acids produced by the gut microbiota are essential in the regulation of glycolipids and energy. Studies have shown that in human primary hepatocytes, bile acids promote the β -oxidation of fatty acids by activating FXR^{18,49} and up-regulate the expression of apolipoprotein CII gene to reduce the triglyceride content in the liver²¹.

As a conjugated bile acid derivative, TUDCA has been shown to treat nonalcoholic fatty liver disease (NAFLD) by acting as an endogenous chemical chaperone; to protect cells against ER stress^{26,50,51} and to reduce liver steatosis⁵². Moreover, TUDCA can alleviate dextran sulfate sodium-induced colitis in mice^{53,54}. *In vitro*, we found that TUDCA could act as an agonist of the Nrf2/ARE signaling pathway. This is reminiscent of the expression pattern of Nrf2, a crucial stress regulator to reduce the ROS, which is a key factor in sensitizing insulin signaling⁵⁵⁻⁵⁷. It has also been confirmed that up-regulation of the Nrf2 expression both by genetic manipulation and pharmacological interference, can significantly decrease the content of ROS and ameliorate IR and T2D^{58,59}. In the mice supplement with metformin, the significant up-regulation in the expression of *Nrf2* and its downstream target genes (*Gclc*, *Cat* and *Ho-1*) occurred, which further alleviated IR and over-oxidation. Conversely, the stress-resistant effect of TUDCA was dramatically repressed by the inhibition of the Nrf2/ARE signaling. *In vitro* experiments have found that TUDCA have similar effects, which can effectively reduce the inflammation and oxidative stress caused by PA as well as decreasing the accumulation of lipid peroxide. This might be a novel mechanism for metformin in the treatment of insulin resistance and diabetes.

In our present study, metabolomics data showed that TUDCA in the intestinal contents of the HFD + Met group increased significantly. Previous studies revealed that metformin treatment increased the levels of the conjugated bile acid in the gut by decreasing the abundance of species of *Bifidobacterium* and its bile salt hydrolase

(BSH) activity in the intestines of individuals with T2D^{17,30,31}. Bile salt hydrolase, secreted by intestinal bacteria, decomposes TUDCA in the intestine and metabolizes TUDCA to taurine and UDCA. However, data from the gut microbiota showed that metformin did not affect the abundance of *Bifidobacterium* and *Lactobacillus*, the major BSH-producing bacteria. In addition, we found that the content of taurine in the intestine increased significantly. *In vitro*, TCDCA can be transformed into TUDCA by 7 α / β -hydroxysteroid dehydrogenase (7 α / β -HSDH)^{15,35,60}. Data from the gut microbiota also revealed that metformin did not affect the abundance of *Eubacterium* and *Clostridium*, the major 7 α / β -HSDH-producing bacteria. Therefore, the increase in TUDCA in the intestine is purportedly not due to the inhibitory effect of metformin on BSH-producing bacteria. Further analysis of the biosynthetic site of bile acids and the liver confirmed that the conjugated bile acid TUDCA was generated in the liver through a series of catalytic reactions and enzymes such as CYP7A1 and BAAT. CYP7A1 is a rate-limiting enzyme that catalyzes the breakdown of cholesterol into bile acids in the liver^{15,61} whereas BAAT is a vital enzyme that catalyzes the amidation reaction of taurine and UDCA^{46,62}. Interestingly, we found that the gene expressions of *Cyp7a1* and *Baat* were significantly enhanced in the HFD + Met group, which might further promote the synthesis of TUDCA in the liver. HPLC analysis also showed an increase in the content of TUDCA in the livers of the HFD + Met group. We suppose that the increase in TUDCA biosynthesis may be the primary cause of the increase in TUDCA in the intestine.

Overall, in our present study, metformin was found to increase the bile acid TUDCA production by increasing bile acid synthase *Cyp7a1* and *Baat*. As an activator of the Nrf2/ARE signaling pathway, TUDCA promotes Nrf2 phosphorylation at Ser40. It also increases the gene transcription of its downstream antioxidant enzyme, reduces oxidative stress and sensitizes insulin signaling; thereby alleviating insulin resistance caused by HFD. The increase of TUDCA levels in the intestine corresponded to the increase in the abundance of *A. muciniphila*. Metformin

remodels the gut microbiota by promoting probiotics such as *Muribacaceae*, and *Parabacteroides*, and inhibiting pathogenic bacteria such as *Erysipelotrichia* and *Helicobacter*. This maintains intestinal integrity and reduces inflammation induced by HFD. However, for a better understanding of the crucial mechanisms involved in the long-term treatment with metformin in humans, the role of the intestine-liver axis should be explored. Since *A. muciniphila* and/or TUDCA are the novel target of metformin in the gut, they are potential targets for the treatment of metabolic conditions in humans.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Y.Z., Y.C., L.Y and J.Z. performed the research. H.T., Y.G. and M.W. designed research. Y.Z., Q.L. and S.W. provided technical assistance. H.T. and Y.Z. analyzed the data. Y.Z. and H.T. wrote the paper. H.T., Y.G. and M.W. had primary responsibility for final content. All authors have read and approved the final manuscript.

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Figure captions

Figure 1. Metformin prevents obesity and systemic metabolic disorders in obese mice induced by HFD. Body weight (A), weight gain (B), food intake (C), H&E staining of epididymal adipose tissue (D, 400×), number of adipocytes (E), levels of total cholesterol and triacylglycerol in liver (F) and serum (G), and serum FFA (H) are shown. Data are expressed as the mean ± SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 2. Metformin improves glucose homeostasis and insulin sensitivity in diet-induced obese mice. IPGTT (A); AUC of IPGTT (B); IPITT (C); slope of IPITT (D) measured at the end of the experimental period. Serum level of FBG (E), FINS (F), and HOMA-IR (G). Protein expression of Akt, phosphorylated Akt, IRS-1, phosphorylated IRS-1 Ser307 and quantitative analysis for the densitometry of pIRS-1 and p-Akt protein level(H). Data are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 3. Metformin blunts intestinal and liver inflammation. Determination of inflammatory factors in liver (A) and ileum (B) by qPCR and serum LPS (C) by kit. Expression levels of IκB, phosphorylated IκB, P65, and phosphorylated P65 as determined by western blot and quantitative analysis for the densitometry of p-IκB and p-P65 protein level (D). Data are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 4. Metformin ameliorates HFD-induced oxidative stress. Representative markers of oxidative stress level: CAT (A), MDA (B), and GSH:GSSG ratio in the liver (C). Protein expression of total Nrf2, phosphorylated Nrf2, total AMPK, and phosphorylated AMPK; quantitative analysis for the densitometry of the p-Nrf2 and p-AMPK protein (D). Quantitative RT-PCR analysis of *Nrf2*, *Keap1* and representative downstream genes (*Nqo1* and *Ho-1*) (E). Data are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 5. Metformin remodels fecal microbial structure and communities.

Microbial composition with cluster at the phylum level (A); relative abundance of gut microbiota at the phylum level (B); ratio of Bacteroidetes to Firmicutes (C); function analysis of gut microbiota (D). Data are represented as mean $\pm$ SD. * $P < 0.05$ and ** $P < 0.01$.

Figure 6. Genus-based comparison of microbial similarities and differences.

Heatmap showing clustering of bacterial communities with their relative abundances at the genus level (A). The bacterial communities or OTUs were clustered phylogenetically by neighbor-joining method and the libraries were clustered by profile pattern using Unifrac analysis. Relative abundances (log values) of microbial genus are displayed in a red-to-blue color code (high to low abundance). Linear discriminant analysis (LDA) score for taxa differing between treatment groups (B). LDA scores with threshold > 3 indicated a higher relative abundance in the corresponding group than in the other two groups.

Figure 7. Differential metabolites determined by non-targeted metabolomics.

Metabolite clustering heat map (A); OPLS-DA score plot (B), in the OPLS-DA score plot, each data point represents one sample, and the distance between points in the plot indicates the similarity between samples; number of different metabolites between each group (C), the major upregulated differential metabolites (D).

Figure 8. Correlation heatmap for gut microorganisms and metabolites.

Matrix heatmap showing the correlation between gut microbiota and metabolome at Phylum (A) and Species (B) levels. Growth curve of *A. muciniphila* in the presence of metformin or TUDCA (C).

Figure 9. TUDCA alleviates insulin resistance caused by PA.

Glucose uptake (A) and the blood glucose remaining in supernatant (B). Protein levels of p-Akt, Akt, pIRS-1 Ser307, IRS-1 were determined by western blot (C). Data are expressed as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 10. TUDCA relieves PA-induced insulin resistance via activating Nrf2/ARE signaling pathway. Intracellular ROS content (A); the ratio of GSH/GSSG (B); Protein levels of p-Nrf2 and Nrf2 were determined by western blot (C); Quantitative RT-PCR analysis of *Nrf2*, representative Nrf2 downstream genes *Ho-1* and *Nqo1* (D); Under Nrf2 knock-down, glucose uptake (E) and blood glucose in the medium supernatant (F) are shown. Data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 11. Metformin increases hepatic TUDCA. The content of TUDCA in the liver was determined using HPLC (A), and the relative mRNA levels of bile acid synthetase (*Cyp7a1*, *Cyp27a1*, *Baat*) determined by qPCR (B).

895 **Table 1. Diversity of gut microbiota in HFD and HFD+Met treated mice**

Groups	Reads	OTUs	Observed species	Ace	Chao1	Shannon	Simpson
NCD	78316±4945	372±35	330±21	345±17	350±21	5.10±0.67	0.92±0.04
HFD	76154±7745	350±40	312±20	325±24	326±24	5.38±0.33	0.95±0.02
HFD+Met	80004±421	374±21	335±20*	342±22*	348±20*	5.24±0.27	0.95±0.02

896 Data are expressed as mean ± SD.

897 * Indicates significantly different from the HFD group at the $*P < 0.05$ level.

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Figure 1

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Figure 3

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