

# Regulation of intestinal micro ecology between raw and salt-processed *Alpinia oxyphylla* on renal injury rats

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**Abstract:** *Alpinia oxyphylla* Fructus is one of the traditional Chinese medicine plants in the treatment of kidney injury. In clinical practice, crude *Alpinia oxyphylla* Fructus (CAOF) and salt-processed *Alpinia oxyphylla* Fructus (SAOF) are the two commonly used drugs specified in the prevention and treatment of diabetic nephropathy (DN). However, the intestinal micro ecology regulation between CAOF and SAOF on DN has not been reported. In this paper, intestinal micro ecology regulation activities between CAOF and SAOF in DN rats were compared and analyzed by short-chain fatty acids (SCFAs) and intestinal flora analysis. The results showed that both SAOF and CAOF can regulate the intestinal flora metabolite SCFAs level in DN rats, reduce blood glucose concentration and improve inflammatory reaction. The intestinal flora analysis showed SAOF and CAOF could increase the intestinal bacterial diversity. The treatment of renal injury may be related to their increased intestinal bacterial diversity.

**Keywords:** *Alpinia oxyphylla*, diabetic nephropathy, intestinal flora, short chain fatty acids.

## INTRODUCTION

The fruit of *Alpinia oxyphylla* (*A. oxyphylla*) is a traditional Chinese medicine which is one of four southern medicines in China. It has medicinal value of anti-uric acid (Li *et al.*, 2016), anti-inflammatory (Thapa *et al.*, 2021), anti-hyperglycemic (Xie *et al.*, 2017). The medicinal part is the seed of *A. oxyphylla* and the crude and salt processed products are commonly used (in) clinically. According to the theories of Traditional Chinese Medicine (TCM), salt processing increased the inclination and direction of AOF's actions, thereby strengthening its efficacy in treatment of renal injury (Sung *et al.*, 2022). The SAOF is more widely used in traditional Chinese medicine prescriptions, such as Suoquan pill, Sanxian pill, which are commonly used in the treatment proteinuria in kidney injury (Shuai *et al.*, 2018). *A. oxyphylla* decoction pieces can be divided into two varieties: CAOF and SAOF. CAOF is obtained by washing the fruit of *A. oxyphylla* and removing the shell. SAOF is made of CAOF is soaked in salt water and fried in low fire.

The research shows that the main effective chemical components of the *A. oxyphylla* extracts were sesquiterpenoids, diterpenes, flavonoids and diarylheptanoids (Li *et al.*, 2021 and Wang *et al.*, 2015). After SAOF treatment, the content of flavonoids in *A. oxyphylla* increased significantly (Ying *et al.* 2016). The extract of *A. oxyphylla* had significant antioxidant and anti-hyperglycemic effects. *A. oxyphylla* extract could significantly improve hyperglycemia and hyperlipidemia

by reducing the blood glucose concentration and oxidative stress, increasing plasma insulin level and improving renal function expression in type II diabetes db/db rats (Du *et al.*, 2017). The extraction of nootkatone from *A. oxyphylla* has anti-inflammatory effect on pbl-2h3 cells (Zhang *et al.*, 2020).

Diabetic nephropathy (DN) is one of the common and serious microvascular complications with diabetes and has been the major cause of death in diabetic patients (Alberto *et al.*, 2015). Once diabetic nephropathy occurred, persistent proteinuria is more irreversible and it gradually evolves to end-stage renal disease (Bonner *et al.*, 2020). Statistics from International Diabetes Federation (IDF) showed that patients with diabetes were more than 450 million in the world in 2017 and it is estimated that the number will reach 629 million by 2045 (Cho *et al.*, 2018). As a typical metabolic disease, its process of occurrence and development includes not only hemodynamic abnormalities, metabolic abnormalities, but also inflammatory reactions, oxidative stress, renin angiotensin system (RAS) activation and gastrointestinal flora imbalance (Lu *et al.*, 2020 and Fernandes *et al.*, 2018). Therefore, study the pathological mechanism and effective methods to prevent of DN have great significance and necessity.

In recent years, the influence of intestinal microflora on diabetes and its complications has become one of the most hotspots. Intestinal flora is a complex microbial community. The number of bacteria in the intestine is approximately 10<sup>14</sup>, (study) about 10 times of the total number of cells in the human body (Meijers *et al.* 2019). The human intestinal microbiota is essential to the health

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of the host and plays a role in pathogen resistance, regulation of immune responses, stabilizing intestinal environment and providing energy metabolites to the host (Huang *et al.*, 2022). The changes of intestinal flora composition and metabolic activity can lead to intestinal flora disorder, which is closely related to the occurrence and development of DN, but the specific mechanism has not been clarified. Recent studies discovered that intestinal flora digests dietary fiber for host to produced short chain fatty acids (SCFAs), such as acetate, butyrate and propionate (Briskey *et al.*, 2017). SCFAs were the main energy resource of intestinal epithelium and provide extra energy for host. SCFAs can also reduce inflammation by inhibiting the activity of inflammatory cells (Li *et al.*, 2019). SCFA acetate can inhibit the histone deacetylase activity of T cells and regulate the oxidative stress of different immune cells. These results suggested that acetate had a good effect of anti-inflammation (Al-Harbi *et al.*, 2018). In addition to kidney disease caused by acetate, feeding a high-fiber diet mice have increased intestinal butyrate and becoming better at infecting *Escherichia coli* (Lénon *et al.*, 2022). Therefore, intestinal flora may be closely related to the occurrence and development of DN.

Based on the pharmacodynamic evaluation of diabetic nephropathy rat models, the modulating effects of CAO and SAOF on intestinal flora of DN rats were determined with 16S rRNA high-throughput sequencing and GC determination of SCFAs content. Through this research, we hope to explore the mechanism of SAOF in improving DN.

## MATERIALS AND METHODS

### *Preparation extract of CAO and SAOF*

The *A. Oxyphylla* were collected from Qiongzong County, Hainan Province in July 2021 and authenticated by Prof. Y.F. Tan (Hainan Medical University, Haikou, Hainan, China).

SAOF was prepared according to the previous report with slight modifications (Gong *et al.* 2016). Briefly, the fruits of *A. oxyphylla* (750g) were broken. The seeds (500g) were sorted out and mixed with 5% saline solution (200mL) for 30 min. Then the seeds were stir-fried at 250°C for 8 min to get SAOF.

CAOF and SAOF (500g) were crushed and extracted twice with 95% (v/v) ethanol (4000mL) under reflux, 2h for each time. And then the extracts were combined and concentrated under reduced pressure at 60°C to dryness. The dried extracts of CAO (44.36g) and SAOF (37.42g) were stored at 4°C for experimental usage.

### *Chromatography analysis of CAO and SAOF*

The contents of nootkatone, oxyphyllacinol and yakuchinone A in the CAO and SAOF were detected by

UFLC-MS/MS. The analysis was carried out as previous method (Li *et al.*, 2013). Briefly, this analysis was executed on a Shimadzu LC-20 AD UFLC system (Shimadzu Corp., Tokyo, Japan), equipment with an AB-SCIEX API 4000+ mass spectrometer (Toronto, Canada). The AB-SCIEX Analyst software packages were used to control the UFLC-MS/MS system, data acquisition and processing. A Phenomenex Kinetex XB-C18 column (2.10mm×50mm, 2.6µm) was employed and column temperature was set at 40°C. The binary phase was composed of A (0.2% formic acid aqueous solution) and B (0.2% formic acid acetonitrile) using a gradient elution as follows: 30% B at 0-1 min, 30-90% B at 1-9 min and 90-30% B at 9.01-10.00 min with a flow rate as 0.4 mL·min<sup>-1</sup>. The mass spectrometer was performed in the positive ion ESI mode with multiple reaction monitoring (MRM) for all the analytes as the same as the analytes in the literature (Li *et al.*, 2013). The capillary voltage and source temperature were set at 5.5 kV and 550°C, respectively.

Twenty-five mg of SAOF extracts were dissolved with 10ml of acetonitrile and diluted 1000-fold with acetonitrile and filtered through a 0.45µm membrane filter before injection. Five µL samples were injected and the contents of nootkatone, oxyphyllacinol and yakuchinone A were tested and calculated.

### *Animals and experimental design*

Male SD rats (200-230g) were SPF grade, purchased from Changsha Tianqin Biotechnology Co., Ltd. This study was conducted in accordance with the Experimental Animal Administration regulations issue by the State Committee of Science and Technology of the People's Republic of China. All procedures described here had prior approval from the Institutional Animal Care and Use Committee at the Huanghe Science & Technology College (Approval number: 2022-004; 9 March 2022). All efforts were made to minimize animal suffering. The rats were maintained at constant temperature of 25°C and 50-60% relative humidity with a 12 h light/dark cycles acclimated for one week before use. Diabetic nephropathy was induced by a single intraperitoneal injection of 50mg/kg STZ (18883-66-4, Shanghai yuanye Bio-Technology Co., Ltd, China) dissolved in 0.1M citrate buffer (pH=4.5) after 12h fasting. One week after the injection, a blood sample was collected from the tail vein to measure the blood glucose level. The rats with a blood glucose levels exceeding 250mg/dL (13.88mmol/L) were considered as diabetic rats (Karatug *et al.*, 2013). The rats were randomly divided into five groups of 7 mice each: (1) intact control (non-diabetic, ND), (2) intact diabetic (STZ), (3) diabetic rats treated with metformin 40mg/kg (STZ-met), (4) diabetic rats treated with CAO 800mg/kg (CAOF), (5) diabetic rats treated with SAOF 800mg/kg (SAOF). CAO and SAOF groups received daily gastric gavage of CAO (800mg/kg) and SAOF (800mg/kg),

respectively. STZ-MET group is received intragastric administration of metformin (40mg/kg) daily and the ND and STZ groups were administered the same amount of distilled water for 4 weeks. At the end of the 4-week period, individual mice were placed in metabolic cages to obtain 24-h urine for the analysis of urine albumin concentration. The fresh feces were collected for high-throughput sequencing of 16S rRNA. The SCFAs contents of feces were determined by gas chromatography (GC). Then, the mice were anaesthetized with sodium pentobarbital, blood samples were collected via femoral artery and then the rats were sacrificed by cervical dislocation. Kidney samples were fixed in 10% neutralized formalin for histology and Immunohistochemistry.

### **Renal histopathology**

Renal tissues were cut into 4mm slices and fixed in 10% paraformaldehyde solution. The tissues were embedded in paraffin and cut into 4mm slices. The paraffin section was stained with hematoxylin-eosin (HE), observed at a Nikon Optical Light Microscope (40×magnification).

Paraffin-embedded kidney sections were used for immunohistochemical staining. After deparaffinization and hydration, the tissue slides antigen was retrieved by citric acid buffer (PH6.0) according to microwave antigen retrieval method. To quench endogenous peroxidase activity, sections were incubated in dark to avoid light in 3% (3mL: 100mL) H<sub>2</sub>O<sub>2</sub> solution for 25 min at room temperature. Nonspecific binding sites were blocked with 3% BSA for 30 min. Sections were then incubated with TGF-β1 (1:500, ab92486, Abcam, UK) overnight at 4°C. Then the slides were washed with tris-buffered saline (TBS, PH7.4) three times and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:15000, 100μl) for 50 min at 37°C. After washed with TBS, the slides incubated with DAB chromogenic agent. The sections were counterstained with Mayer's haematoxylin, dehydrated, mounted and observed on a Nikon Optical Light Microscope (40×magnification).

### **Biochemical analysis**

The collection of 24h urinary protein of all rats was detected and centrifuged at 3000 rpm for 5 min. Urinary protein concentrations were measured by urine protein test kit. Fasting blood glucose (FBG) concentrations were determined using blood glucose meter. The blood samples were centrifuged at 7000rpm for 10min at 4°C to obtain the serums. Serum creatinine (Scr), Blood urea nitrogen (BUN), Triglycerides (TG), Total cholesterol (TC), High density lipoprotein cholesterol (HDL-C) and Low density lipoprotein cholesterol (LDL-C) in serum were determined by the commercial kit. All of the procedures were carried out according to the manufactures' instructions.

### **Short-chain fatty acids analysis**

The contents of fecal acetate, propionate and butyrate levels were measured by GC, according to the previous literature with minor modification (Wang *et al.* 2012). Briefly, 200 mg of fecal sample was weighted, suspended in 4 mL of sterile water and oscillated for 20 min at room temperature. The suspension was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant (900μL) were spiked with the internal standard, 2-ethylbutyric acid (100μg/ml, 100μL, Aladdin, China). The samples (1μL) were then injected into a Thermo TRACE 1300 gas chromatography equipped with a flame ionization detector (FID). A gas chromatographic column (HP-FFAP, 30m×0.25 mm×0.25μm, Agilent) was used in this analysis. Nitrogen was used as the carrier gas. The injection inlet temperature is 280°C. The initial column temperature was maintained at 100°C for 3 min. The temperature was raised to 160°C at 3°C/min and held for 3 min, then raised to 190°C at 2°C/min and held for 5 min. The temperature of FID was set as 300°C. The injection volume is 2μL.

### **Diversity analysis of intestinal bacteria**

After treatment, 4 rats were randomly selected from each group and transferred to fresh sterilized cages. The feces of each rat were respectively collected and immediately stored at -80°C for subsequent DNA extraction. The total DNA was extracted from 200 mg samples using the Power Soil® DNA Isolation Kit (12888-50, Mobio, USA) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the V4 regions of bacterial 16S rRNA genes was performed using universal primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR reaction conditions are as follows: pre-denaturation at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 57°C for 45 s and 34 cycles of extension at 72°C for 1 min, 72°C extension at 10 min, finally 16°C for 5 min, storage at 4°C. The PCR products were detected by electrophoresis on 2% agarose gel. After the PCR products were quantitatively mixed, the library was constructed using the TruSeq® DNA PCR-Free Sample Preparation Kit. The constructed library was quantified by Qubit and Q-PCR. The Illumina novaseq 6000 PE250 is used for on-machine sequencing.

Use Uparse software (Uparse v7.0.1001, <http://www.drive5.com/uparse/>) to cluster all Effective Tags of all samples. By default, the sequence is clustered with 97% identity Become Operational Taxonomic Units (OTUs). Use Mothur method and SILVA132 (<http://www.arb-silva.de/>) SSUrRNA database to perform species annotation analysis (set the threshold to 0.8~1). OUT taxonomies (ranking from phylum to species) were determined based on the database of National Center for Biotechnology Information (NCBI).

## STATISTICAL ANALYSIS

Statistical Package for the social sciences (SPSS), version 20 (SPSS Inc. Chicago, IL, USA) was used for statistical analysis. Data were evaluated by one-way analysis of variance (ANOVA) along with Tukey HSD. Values were considered significantly different when \* =  $p < 0.05$  (significant), \*\* =  $p < 0.01$  (more significant), \*\*\* =  $p < 0.001$  (highly significant).

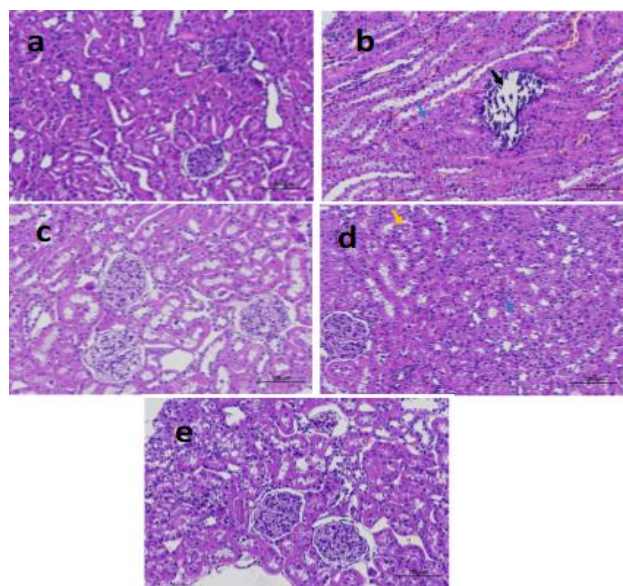
## RESULTS

### Quantitative analysis of CAOF and SAOF

The contents of nootkatone, oxyphyllacinol and yakuchinone A in CAOF and SAOF are shown in table 1. It can be seen that there are great differences in the content of chemical components between CAOF and SAOF. The contents of three chemical components decreased greatly after salt-processed.

**Table 1:** The contents of three compounds in SAOF and CAOF.

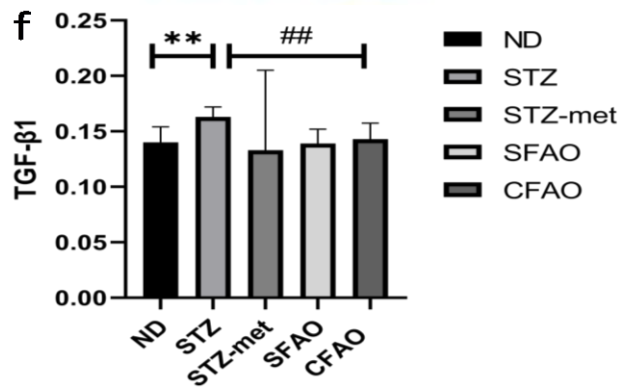
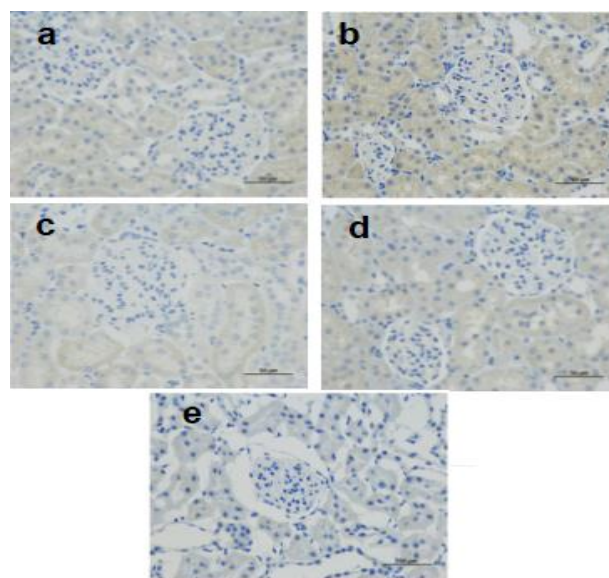
Compounds	CAOF	SAOF
Yakuchinone A	1.977	0.150
Oxyphyllacinol	1.993	0.116
Nootkatone	16.491	3.883



**Fig. 1:** Histological study of H&E staining in rat kidney sections. (a) normal group; (b) STZ-induced diabetic rats group: renal tubular necrosis calcifications (black arrow); a small amount of renal tubular epithelial cell occurred granule degeneration (blue arrow); (c) Metformin-treated drug group; (d) SAOF treatment (800mg/kg) group: a small amount of renal tubular epithelial cell occurred granule degeneration (blue arrow) was seen and the cytoplasm was loosely and lightly stained, a small amount of renal tubule brush edge fallen off (yellow arrow). (e) CAOF treatment (800mg/kg) group.

### Renal histopathology

As shown in fig. 1a, normal rats have clear layers of renal structures, stained evenly and with regular glomerular shape. Renal tubular epithelial cells were lined up tightly and no obvious inflammation was observed. While in STZ group, necrosis calcification of renal tubules occurred (black arrow); a small amount of renal tubular epithelial cell occurred granule degeneration (blue arrow) in fig. 1b. In the SAOF group, a small amount of renal tubular epithelial cell occurred granule degeneration (blue arrow) (was seen) and the cytoplasm was loosely and lightly stained, a small amount of renal tubule brush edge fallen off (yellow arrow) (fig. 1d). There was no obvious abnormality in the glomerular morphological structure of the CAOF group, there was no obvious inflammatory cell infiltration (fig. 1e).



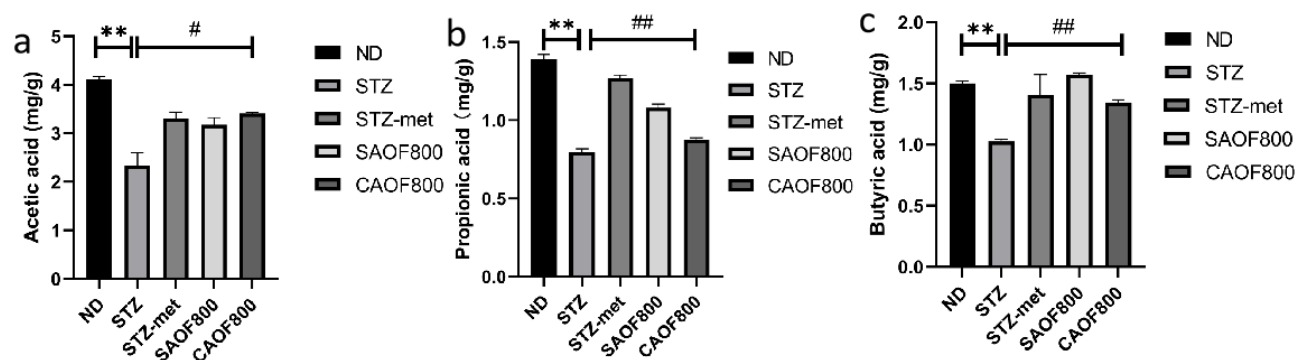
**Fig. 2:** The expression of TGF- $\beta$ 1 in the kidney tissue of different groups is represented. (a) Control, (b) STZ, (c) STZ-met, (d) SAOF 800 (e) CAOF 800 Control= rats fed a normal diet and water; STZ = STZ-induced diabetic rats group; STZ-met= Metformin-treated drug group; SAOF = SAOF treatment (800mg/kg) group; CAOF30=CAOF treatment (800mg/kg) group. Compared with the ND group, \*\* $P < 0.01$ ; Compared with the STZ group, ## $P < 0.01$ .

**Table 2:** Comparison of 24h renal function parameters in rats (mean±SD, n=7).

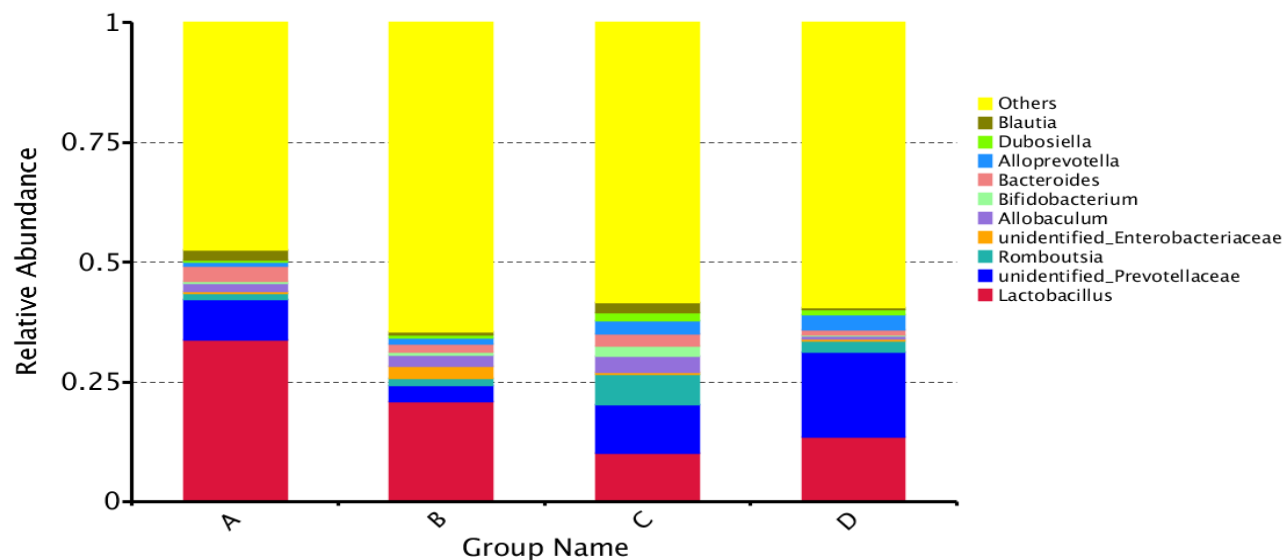
	ND	STZ	STZ-met	SAOF	CAOF
Urine protein (mg/L)	673.26±238.87	2135.30±317.87*	150.72±20.65##	374.29±57.32##	478.52±54.42##
Scr (umol/L)	35.30±5.76	40.42±11.54	30.37±2.42#	36.75±6.36	27.36±5.12##
BUN (mmol/L)	4.50±0.63	5.96±1.33*	4.48±0.76##	4.45±0.72##	4.68±0.72#
TG (mmol/L)	0.30±0.10	1.29±0.29**	0.33±0.11##	0.46±0.14#	0.31±0.06#
TC (mmol/L)	2.83±0.24	3.56±0.29*	2.83±0.59	2.50±0.23##	2.43±0.30##
HDL-C (mmol/L)	3.30±0.97	1.89±0.41**	3.04±0.86##	2.46±0.38	2.70±0.64##
LDL-C (mmol/L)	0.51±0.39	1.76±0.24**	0.82±0.32##	0.58±0.53##	0.83±0.17##

Compared with the normal group, \*P<0.05, \*\*<0.01.

Compared with the STZ group, #P<0.05, ##P<0.01.



**Fig. 3:** Effects on short-chain fatty acid levels of each groups, feeding DN rats with CAOF and SAOF increased the content of SCFAs in fecal intestinal metabolites. (a) acetic acid; (b) propionic acid; (c) butyric acid; Compared with the ND group, \*\*P<0.01; Compared with the STZ group, #P<0.05, ##P<0.01.



**Fig. 4:** Relative abundance of microbial species at the Genus level in the feces of rats. (A) normal group; (B) STZ-induced diabetic rats group; (C) Metformin -treated drug group; (D) SAOF treatment (800 mg/kg) group; (E) CAOF treatment (800 mg/kg) group.

Immunohistochemical staining method was employed to detect the expression of fibrosis-related protein TGF- $\beta$ 1 in renal tissue (fig. 2). Compared with the ND group, the expression of TGF- $\beta$ 1 was up-regulated in the STZ group ( $P<0.01$ ). After treated with CAOF and SAOF, the TGF- $\beta$ 1 level in the kidney was significant reduced in fig. 2.

#### Biochemical analysis

As shown in table 2, the urine protein level in the STZ group was significantly increased ( $P<0.01$ ) compared with the ND group. The treatment with CAOF and SAOF groups could significantly reduce the urine protein level ( $P<0.01$ , table2).

The levels of Scr, Bun, TG, TC and LDL-C were significantly increased in STZ group compared with the ND group. The BUN, TG, TC, LDL-C in plasma of SAOF group significantly decreased compared with the STZ group ( $P < 0.01$ ). The Scr, BUN, TG, TC, LDL-C in plasma of CAOF group significantly decreased and HDL-C significantly increased compared with the STZ group ( $P < 0.01$ ,  $P < 0.05$ ).

#### **Quantitative analysis of SCFAs**

The concentrations of acetic acid, propionic acid and butyric acid in fecal were determined by GC. As shown in fig. 3, the rats with diabetes showed decreased concentrations of acetic acid, propionic acid and butyric acid compared with the control rats ( $P < 0.01$ ). The rats treated with SAOF and CAOF resulted in significantly increased SCFAs levels ( $P < 0.05$ ,  $P < 0.01$ ), butyric acid concentration increased by 50% in SAOF group.

#### **Analysis of intestinal flora**

High-throughput sequencing of the V4 regions of the 16S rDNA gene was used to analyze the structural changes of gut microbiota in DN mice treated with CAOF and SAOF for 4 weeks. After 4 weeks of SAOF and CAOF intervention, the intestinal flora diversity was significantly increased in the SAOF and CAOF group compared with the STZ group (fig. 4). The Romboutsia and Prevotellaceae levels of the STZ group were significantly decreased compared with the ND group. After treated with CAOF and SAOF, the Romboutsia and Prevotellaceae relative abundance level could significantly increase compared with the STZ group.

## **DISCUSSION**

DN is one of the common complications of diabetes mellitus and its pathogenesis is still unclear. Intestinal flora is the largest micro ecosystem in the human body, which participates in the metabolism of substances and energy in the body. Recent studies have revealed that in addition to obesity, genetic and insulin dysfunction, intestinal flora disorders may also be an important cause of DN (Sheng *et al.*, 2018). Transforming growth factor- $\beta 1$  (TGF- $\beta 1$ ) is an important promoting fibrosis factor, which mediates renal fibrosis and glomerulosclerosis. Continuous hyperglycemia can produce TGF- $\beta 1$  by stimulating the kidney of DN, which increases the expression and secretion of extracellular matrix protein (ECM) and leads to renal fibrosis (Hills *et al.*, 2018 and Yoon *et al.*, 2018). This study showed that the expression of protein TGF- $\beta 1$  was significantly increased in renal tissue of DN. The DN rats treated with CAOF and SAOF significantly inhibited the up-regulation of TGF- $\beta 1$ , indicates that CAOF and SAOF may have a potential effect in reducing renal fibrosis of DN.

Intestinal microflora is one of the main research hotspots at present. The homeostasis of various microorganisms in

the intestine is one of the important reasons to maintain the homeostasis of physiological function. The disorder of intestinal flora will cause many problems in nervous system, immunity and metabolism. A recent experiment shows that the intestinal microflora of DN rats changed from phylum to genus classification and the ratio of Firmicum /Bacteroides in intestinal bacteria of diabetic rats decreased, while some pathogenic bacteria (Clostridium and Escherichia coli) increased significantly and the variation degree of bacterial flora was closely related to blood glucose concentration (Gilbert *et al.*, 2018). In this paper, experimental studies have shown that after 4 weeks of administration with SAOF and CAOF, the intestinal flora Proteobacteria of DN rats decreased at the phylum level. It is suggested that SAOF and CAOF can reduce the abundance of Proteobacteria to a certain extent.

SCFAs are produced by the intestinal flora through the fermentation of dietary fiber and participate in the metabolism of energy in the body. When the intestinal flora is imbalanced, short-chain fatty acids are lacked, insulin resistance occurs and then the diabetic nephropathy will occur (Huang *et al.*, 2018). Therefore, when the intestinal homeostasis is destroyed, the intestinal flora will affect the content of short-chain fatty acids, which in turn affects the host's energy metabolism, insulin resistance and the development of diabetes. The main SCFAs in intestinal tract were acetic acid, propionic acid and butyric acid, accounting for more than 95% of all SCFAs (Sun *et al.*, 2017). Previous studies have shown that butyrate, propionate and acetate can prevent obesity and insulin resistance induced by high fat in mice. Acetate and propionate are important substrates in the process of gluconeogenesis and adipogenesis. Acetate can stimulate islet  $\beta$ -cells to secrete insulin and propionate acid can inhibit the synthesis of cholesterol and reduce blood lipids (Bridgeman *et al.*, 2022). It has been reported that butyrate is an anti-inflammatory short chain fatty acid salt, which can provide nutrition for intestinal mucosa and enhance intestinal mucosal immunity (Gasaly *et al.*, 2021 and He *et al.*, 2022). Thus it is shown that diabetic nephropathy cause a certain degree of intestinal flora imbalance.

## **CONCLUSION**

The present experimental research explains that CAOF and SAOF may have a potential effect in reducing renal fibrosis of DN. And our research also suggests that SAOF and CAOF can reduce the abundance of Proteobacteria to a certain extent.

## **DECLARATION OF COMPETING INTEREST**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

## ACKNOWLEDGEMENTS

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## ETHICS STATEMENT

All the animal experiments was conducted in accordance with the Experimental Animal Administration regulations issue by the State Committee of Science and Technology of the People's Republic of China and prior approval from the Institutional Animal Care and Use Committee at the Huanghe Science & Technology College (Approval number: 2022-004).

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