

RESEARCH ARTICLE



## TangNaiKang, herbal formulation, alleviates obesity in diabetic SHR/cp rats through modulation of gut microbiota and related metabolic functions

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### ABSTRACT

**Context:** Tangnaikang (TNK) is a Chinese herbal formulation that has lipid-lowering effects, but its effect on reducing obesity has not been studied.

**Objective:** To observe the effect of TNK on obesity and explore its effect on gut microbiota of obese rats.

**Materials and methods:** The SHR/NDmcr-cp rats were divided into three groups: (1) 3.24 g/kg TNK (High TNK), (2) 1.62 g/kg TNK (Low TNK), and (3) an untreated control (CON). Wistar-Kyoto rats were used as normal controls (WKY). After 8 weeks of TNK oral administration, body weight, abdominal circumference, triglycerides (TC) and total cholesterol (CHO) were measured. Gut microbiota diversity was studied by 16S rDNA sequencing, and metagenomes analysis was conducted to determine alteration in functional gene expression.

**Results:** The body weight ( $496.60 \pm 6.0$  g vs.  $523.40 \pm 5.6$  g), abdomen circumference ( $24.00 \pm 0.11$  cm vs.  $24.87 \pm 0.25$  cm), TC ( $3.04 \pm 0.16$  mmol/L vs.  $4.97 \pm 0.21$  mmol/L), CHO ( $2.42 \pm 0.15$  mmol/L vs.  $2.84 \pm 0.09$  mmol/L) of rats in the High TNK group were decreased significantly (all  $p < 0.05$ ). TNK administration regulates intestinal flora, up-regulates *Eisenbergiella* and down-regulates *Clostridium\_sensu\_stricto\_1*, which is beneficial to the production of short-chain fatty acids (SCFAs). Metagenomes analysis shows that TNK is closely related to the fatty acid synthesis pathway.

**Discussion and conclusions:** TNK can regulate gut microbiota to reduce obesity, which may be related to fatty acid metabolism. Our research supports the clinical application of TNK preparation and provides a new perspective for the treatment of obesity.

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### KEYWORDS

Chinese medicine; lipid; diabetes mellitus; metabolic disorder; intestinal microbiota

### Introduction

Obesity is one of the major public health problems the world is facing today. Multiple factors, including genetics, environment, and an imbalance in energy intake and expenditure, determine the risk of developing obesity. Over time, individuals with obesity show pathological changes in multiple organs, e.g., liver, muscle, and even the brain. Studies have shown that obesity is closely associated with metabolic disorders, including hyperglycaemia, insulin resistance, dyslipidemia, hypertension (Bays and Dujovne 2006), and chronic inflammation (Saltiel and Olefsky 2017). Thus, obesity is a complex disease involving multiple signalling pathways with no clear prognosis. With the advent of 16S rDNA sequencing, an increasing number of studies have reported the correlation between gut microbiota and obesity (Clooney et al. 2016). Commensal bacteria can affect their host by various mechanisms including the production or consumption of metabolites. Therefore, the gut microbiota has become an important mechanism affecting obesity.

TangNaiKang (TNK), an herbal formulation, contains five herbal plant extracts: Fructus Ligustri Lucidi [*Ligustrum lucidum* Ait. (Oleaceae)], Prunellae Spica [*Prunella vulgaris* L. (Labiatae)], Rhizoma Saururi [*Saururus chinensis* (Lour.) Baill.



(*Saururaceae*)], Folium Psidii Guajavae [*Psidium guajava* L. (Myrtaceae)] and Radix Ginseng [*Panax ginseng* C. A. Mey. (Araliaceae)]. Its effect on pre-diabetes has been partly explored in some early research in the field (Li et al. 2015). Studies have shown that TNK can decrease lipid levels (Li et al. 2015), decrease blood sugar levels, and reduce insulin resistance (Li et al. 2017). However, its role in reducing obesity has not been studied.

This study investigates whether TNK administration reduces obesity in the SHR/cp rats and determines its effects on gut microbiota.

### Materials and methods

#### Preparation of TNK formulation

TNK contains Fructus Ligustri Lucidi, Prunellae Spica, Rhizoma Saururi, Folium Psidii Guajavae and Radix Ginseng in the 4:4:2:2:1 proportion, respectively. The first four plants were mixed and extracted twice with refluxing 75% (v/v) ethanol (1:8 w/v) for 1 h; then, the obtained solution was concentrated into an ethanol extract with a recovery rate of 13.83%. Afterward, the obtained residue was decocted twice with water (1:6 w/v) for 1 h,

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and the obtained solution was concentrated into a water extract with a recovery rate of 5.49%. To dry the Radix Ginseng, the plants were baked at 60 °C for 4 h, and the dried Radix Ginseng was crushed to yield a fine powder ( $150 \pm 6.6 \mu\text{m}$ ). Last, all components were mixed in a homogenous manner according to the following weight ratio: ethanol extract/water extract/Radix Ginseng fine powder/excipients = 25/10/15/10 (Liu 2004). All crude drugs were purchased from Shenyang Pharmaceutical Group Corporation (Shenyang, China) and authenticated following the Pharmacopoeia of the People's Republic of China (2000 ed).

In our previous study (Li et al. 2015), we used spectrophotometry analysis and found that the content of total flavonoids and triterpenoid saponins in ethanol extract was 4.23% and 3.67%, respectively, while the content of total polysaccharides in water extract was 5.83%. The contents of Rg1, Re, Rb1, and rosmarinic acid in TNK were determined by high-performance liquid chromatography (HPLC) to be 0.09%, 0.12%, 0.10%, and 0.55%, respectively.

### Animals and treatment procedure

#### Animals

SHR/cp rats (Japan SLC, Inc., Shizuoka, Japan), 7-week-old males with the body weights of 190–210 g at arrival, were used. Wistar Kyoto (WKY) rats (Japan SLC, Inc., Shizuoka, Japan), 7-week-old males with body weights of 150–170 g at arrival, were used. Animals were kept in specific-pathogen-free (SPF) animal rooms at the Mukogawa Women's University, where the room temperature was maintained at 22°–24 °C, and the humidity was maintained at 40%–60%. Rats had free access to normal chow and water throughout the experiment. This study was conducted following the Guidelines for the Care and Use of Laboratory Animals at the Mukogawa Women's University. All animal protocols were approved by the Animal Care and Use Committee of Mukogawa Women's University (grant number: P-06-2018-01-A; date of approval: 27 November 2018).

#### Grouping and drug administration

The SHR/cp rats were chosen according to the levels of fasting blood glucose (FBG) and body weight. We use PASS15 software to conduct an F-test analysis on the pre-experimental data and calculate the required sample size. The rats were randomly divided into three groups ( $n=8$ ): (1) Rats treated with 3.24 g/kg TNK (High TNK group), (2) rats treated with 1.62 g/kg TNK (Low TNK group), and (3) an untreated control group (CON). Age-matched male WKY rats were used as normal controls (WKY group). TNK was prepared in sterile water and administered once daily by gastric gavage for eight consecutive weeks. The CON group and the WKY group received the same volume of sterile water. The rats were given clean bedding every two days. Feed and water shall be supplemented according to the water and food inflow measured every week.

#### Other treatments

At the end of the 8th week, the abdominal circumference was measured. The body mass index (BMI) of each rat was measured by bioelectrical impedance analysis (BIA) (ImpediVET, ImprdiMed Ltd., Brisbane, Australia). The rats were placed in the metabolic cage for 24 h to observe their food intake, water intake, and urine volume.

### Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was conducted at the end of the 4th week. The rats fasted for 12 h. Glucose was intragastrically administered at a dose of 2 g/kg. We collected the tail vein blood of the rats at the time points of 0 min before gavage and 30, 60, 90, and 120 min after gavage, then detected the blood glucose (BG) using a blood glucose kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and drew a line diagram according to the blood glucose value in different periods. The area under curve (AUC) was then calculated using the following method:

$$\begin{aligned} \text{AUC} = & 0.25 \text{ BG } 0 \text{ min} + 0.5 \text{ BG } 30 \text{ min} \\ & + 0.5 \text{ BG } 60 \text{ min} + 0.5 \text{ BG } 90 \text{ min} \\ & + 0.25 \text{ BG } 120 \text{ min} \end{aligned}$$

### Sample collection

After 8 weeks of TNK administration, the rats were sacrificed following fasting for 12 h. After anaesthesia, blood was taken through the abdominal aorta. After blood collection, the rats lost blood and died. The whole blood was centrifuged after resting at room temperature for 2 h, and the upper serum was taken for direct detection or frozen at  $-80^\circ\text{C}$  for subsequent detection. The rectal contents of the rats were collected in a sterile environment. Fresh faecal samples were immediately stored at  $-80^\circ\text{C}$  for subsequent analysis.

### Serum biomarker measurement

The serum FBG, total cholesterol (CHO) and triglycerides (TG) were detected using a special kit according to the manufacturer's instructions (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The enzyme method is used for detection, the absorbance of the reaction working solution is detected by an enzyme labeling instrument, and the final result is analysed by analyzing the absorbance data.

### DNA extraction

PCR amplification targeting the V3-V4 hypervariable region of the 16S rDNA of bacteria was conducted, using two universal bacteria 16S rDNA primers: forward CCTAYGGGRBGCASCAG and reverse GGACTACNNGGGTATCTAAT. PCR products were purified with agarose gel electrophoresis, and a gene library was constructed using a TruSeq™ DNA Sample Prep Kit. Sequencing was performed using the Illumina Miseq system (Illumina, USA), based on instructions in the manual.

### 16s rDNA sequencing and bacterial abundance analysis

Raw data from the sequencing was fed into FLASH software (version 1.2.7) for paired-end reads assembling, then quality filtered by QIIME (version 1.7.0) and USEARCH (version 7.0) tools, as described in previous studies (Lozupone and Knight 2005; Magoc and Salzberg 2011; Consortium THMP 2012). Outcoming clean tags were clustered with USEARCH (version 7.0) at a 97% similarity level, and operational taxonomic units (OTUs) were finally generated. The OTUs were then identified into different taxonomy by sequence alignment using an RDP Classifier (version 2.2) with a confidence level of 0.7. Taxonomy reference was downloaded from Silva website (<http://www.arb-silva.de>, Release 132).

Bacteria abundance of the OTUs was calculated by dividing the clean tags from the total clean tags.

### Diversity analysis and differential analysis

Alpha ( $\alpha$ ) diversity of each sample, including Ace, Chao1, Simpson, and Shannon index, was transformed from the OTU list by QIIME (version 1.7.0) tools. For  $\beta$  diversity analysis, the OTU table of all samples was computed as beta diversity metrics, in weighted UniFrac distances, to conduct principal coordinate analysis (PCoA) with QIIME (version 1.7.0) tools. The  $\alpha$  significant value for the non-parametric test among groups was set at 0.01 for more confidence in the results; the threshold on the logarithmic LDA score was set at two, and other parameters were all set as default (Segata et al. 2011).

### Metagenomes prediction and analysis

PICRUSt software predicts metagenome functional content from the 16S rRNA gene (Langille et al. 2013). According to the literature, the predicted metagenomes are highly consistent with sequenced metagenomes. We used this software to calculate the metagenomes of each sample. Next, the metagenomes were collapsed into the KEGG pathway of Level 3 and analysed using the R software (version 3.5.0).

### Statistical analysis

Statistical analysis was performed by SPSS 20.0 (IBM Corp., Armonk, N.Y., USA). One-way analysis of variance (ANOVA) was used to evaluate the differences between groups.  $P < 0.05$  was considered statistically significant.  $P < 0.01$  was considered statistically significant. The genus abundance and metagenome data were compared through ANOVA as well.

## Results

### TNK formulation reduces obesity and hyperlipidaemia in the SHR/cp rats

To investigate whether TNK formulation reversed obesity in SHR/cp rats, we administered TNK formulation to SHR/cp rats for eight weeks, and serum levels of lipids were measured. We found that the body weight (CON  $523.40 \pm 5.67$  g vs. WKY  $408.0 \pm 3.78$  g,  $p < 0.001$ ), BMI (CON  $28.04 \pm 0.53$  g/cm<sup>2</sup> vs. WKY  $21.58 \pm 0.26$  g/cm<sup>2</sup>,  $p < 0.001$ ), abdomen circumference (CON  $24.87 \pm 0.25$  cm vs. WKY  $19.58 \pm 0.25$  cm,  $p < 0.001$ ), triglycerides (CON  $4.97 \pm 0.21$  mmol/L vs. WKY  $0.36 \pm 0.02$  mmol/L,  $p < 0.001$ ) and cholesterol (CON  $2.84 \pm 0.09$  mmol/L vs. WKY  $2.31 \pm 0.14$  mmol/L,  $p < 0.01$ ) were significantly upregulated in the CON group (Figures 1 and 2). Compared with the CON group, the body weight (TNKH  $496.60 \pm 6.0$  g vs. CON  $523.40 \pm 5.6$  g,  $p < 0.01$ ), BMI (TNKH  $26.47 \pm 0.38$  g/cm<sup>2</sup> vs. CON  $28.04 \pm 0.53$  g/cm<sup>2</sup>,  $p < 0.05$ ), abdominal circumference (TNKH  $24.00 \pm 0.11$  cm vs. CON  $24.87 \pm 0.25$  cm,  $p < 0.01$ ), TC (TNKH  $3.04 \pm 0.16$  mmol/L vs. CON  $4.97 \pm 0.21$  mmol/L,  $p < 0.001$ ) and CHO (TNKH  $2.42 \pm 0.15$  mmol/L vs. CON  $2.84 \pm 0.09$  mmol/L,  $p < 0.05$ ) of the TNKH group decreased significantly, and the body weight (TNKL  $498.00 \pm 10.80$  g vs. CON  $523.40 \pm 5.6$  g,  $p < 0.05$ ) and TC (TNKL  $3.47 \pm 0.49$  mmol/L vs. CON  $4.97 \pm 0.21$  mmol/L,  $p < 0.05$ ) of the TNKL group decreased significantly (Figure 2).

### TNK formulation reduces the blood glucose in the SHR/cp rats

The serum level of FBG, OGTT, and AUC<sub>OGTT</sub> were also investigated. The results showed that after 8 weeks of TNK formulation, the FBG level was significantly lower in the High TNK group compared with the level in the CON group (TNKH  $13.22 \pm 0.51$  mmol/L vs. CON  $16.70 \pm 0.71$  mmol/L,  $p < 0.01$ , Figure 5(e)). After 4 weeks of TNK treatment, OGTT was carried out. The results showed that compared with the WKY group, the blood glucose in the CON group increased significantly at 0, 30, 60, 90, and 120 min (Figure 2(c)). Compared with the CON group, the blood glucose of 5-time points of the High TNK group decreased significantly (Figure 2(c),  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ ). The AUC<sub>OGTT</sub> in the TNK groups were significantly reduced (TNKH  $423.5 \pm 15.11$  mmol/L vs. CON  $510.5 \pm 15.96$  mmol/L,  $p < 0.001$ ; TNKL  $433 \pm 17.11$  mmol/L vs. CON  $510.5 \pm 15.96$  mmol/L,  $p < 0.01$ , Figure 2(d)) compared with the CON group. Consistent with a previous report, these results suggest that TNK formulation increases insulin sensitivity.

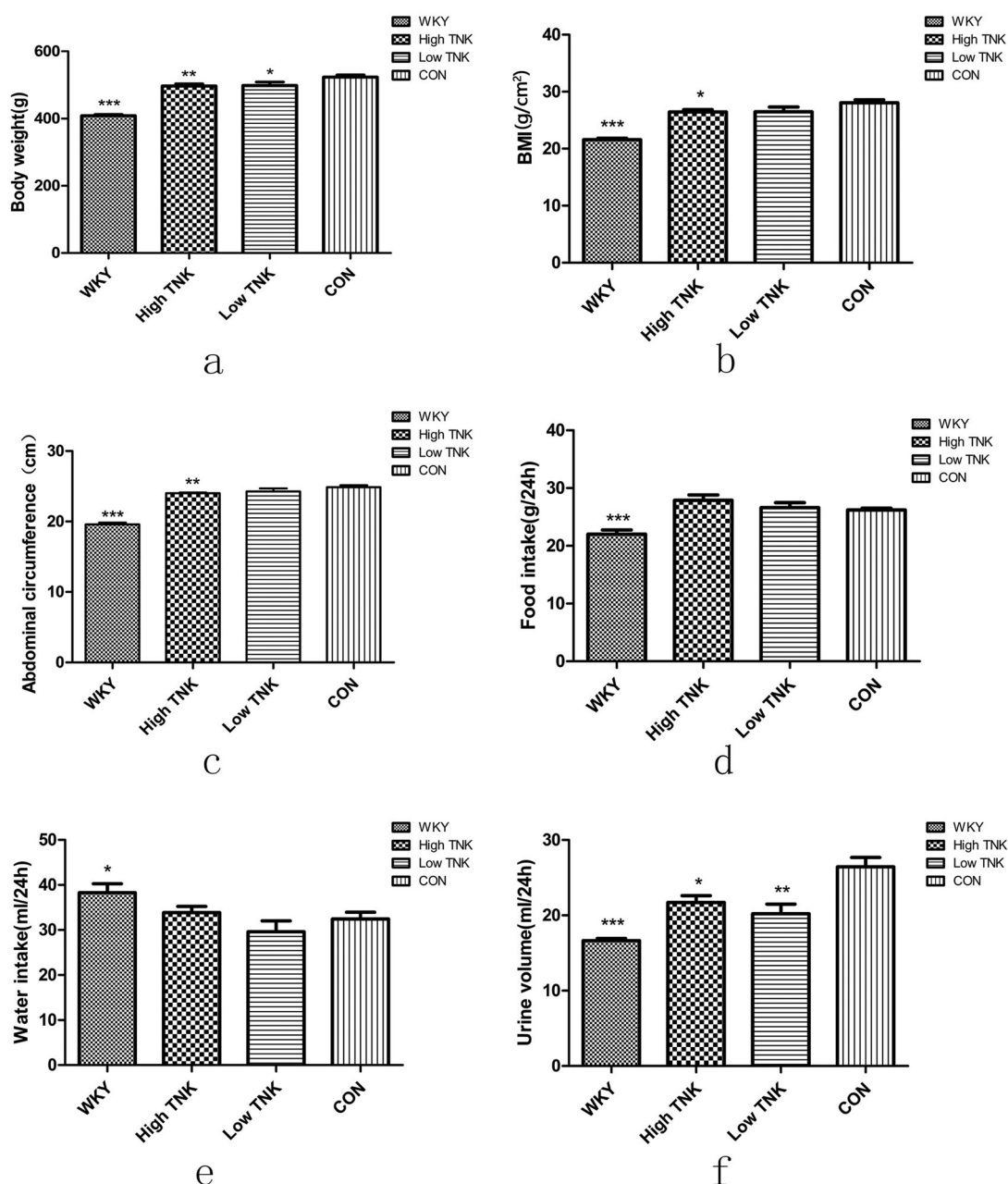
### TNK formulation attenuates gut microbiota damage in SHR/cp rats

We wanted to determine whether administering the TNK formulation would lead to changes in the gut microbiota. To investigate this, we looked at the inner structure of the microbial community under different treatments. A total of 753 OTUs, which represent 865 ecological groups, were generated by paired-end sequencing. Next, the OTUs were divided into 10 phyla and 123 genera, and ecological diversity indexes such as ACE, Chao1, and Simpson and Shannon index were calculated in each sample. There are significant changes between WKY and CON groups, whereas no significant changes were found between other groups (Figure 3). However, we found detectable changes by PCoA analysis based on the OTU table. In this part, we used weighted UniFrac distance, a general type of distance that is used to distinguish different groups. The microbiota profile was separated into unweighted UniFrac distance between the WKY group and the CON group (Figure 4). Since unweighted UniFrac distance is based on sequence similarity, it means that the CON group had a significantly changed microbial community (P-value). In addition, the High TNK group had a markedly changed microbial community, while the change in the Low TNK group was not notable.

Our results showed that the number of bacterial species increased in the rats in the CON group. Interestingly, after the administration of TNK formulation, the number of bacterial species decreased. Thus, next, we analysed the gut microbiota from phyla, including the Actinobacteria, Bacteroidetes, Cyanobacteria, Deferribacteres, Deinococcus-Thermus, Firmicutes, Lentisphaerae, Proteobacteria, Tenericutes, and Verrucomicrobia.

We observed that in contrast with the WKY, the CON group had a greater abundance of Firmicutes ( $p < 0.05$ ) and a lower abundance of Verrucomicrobia ( $p < 0.05$ ). There was also a lower abundance of Bacteroidetes in the CON group as compared to the WKY group, though the difference was not significant. Interestingly, administration of TNK formulation did not significantly change the counts of Firmicutes and Verrucomicrobia. These results suggest that TNK administration might not alter the composition of the gut microbiota in obese animals.

At the levels of bacterial families, 45 families were identified from 22 rat faecal samples; among them, the abundance of 15 OTUs was altered in the CON group. Following administration of



**Figure 1.** After eight weeks of treatment, the body weight, BMI, abdominal circumference, food intake, water intake, and urine volume of rats in each group.

the high dose of TNK formulation, the abundance of 17 OTUs was either enhanced or reduced. As shown in Figure 5(a), the top ten families in rat gut microbiota are Uminococcaceae, Lachnospiraceae, Muribaculaceae, Lactobacillaceae, Akkermansiaceae, Bacteroidaceae, Prevotellaceae, Peptostreptococcaceae, Desulfivibrionaceae, and Erysipelotrichaceae. Among these families, the abundance of Akkermansiaceae was lower in the CON group than in the WKY. However, after TNK formulation treatment, the abundance of Akkermansiaceae did not significantly increase relative to the CON group. The abundance of Clostridiaceae\_1, Erysipelotrichaceae, Defluviitaleaceae, and Family\_XIII was higher in the CON group than that in the WKY; however, Clostridiaceae\_1 showed a decrease in abundance after TNK formulation treatment (Figure 5(b,c), Table 1).

At the levels of bacterial genera, the higher abundance of *Clostridium\_sensu\_stricto\_1* ( $p < 0.05$ ) and lower abundance of *Eisenbergiella* spp. ( $p < 0.05$ ) were observed compared to the

abundance in the CON group. Administration of TNK formulation ameliorated these changes in the CON group (Figure 5(d,e)). *Clostridium\_sensu\_stricto\_1* spp., which is closely connected with inflammation (Xu et al. 2016), showed a high abundance in obesity and type 2 diabetes mellitus (T2DM) patients. Importantly, *Clostridium\_sensu\_stricto\_1* is related to short-chain fatty acids (SCFAs) (Fan et al. 2017), while *Eisenbergiella* may play a key role in nutrients absorbing and restoring the gut barrier. These results suggest the possibility that TNK formulation may mediate its anti-obesity and metabolic disorder effect through modulation of gut microbiota.

#### TNK formulation changes the metabolic genes in metagenomes of the SHR/cp rats

It is considered that metagenomes are the basis for the metabolic function of gut microbiota. As TNK formulation significantly



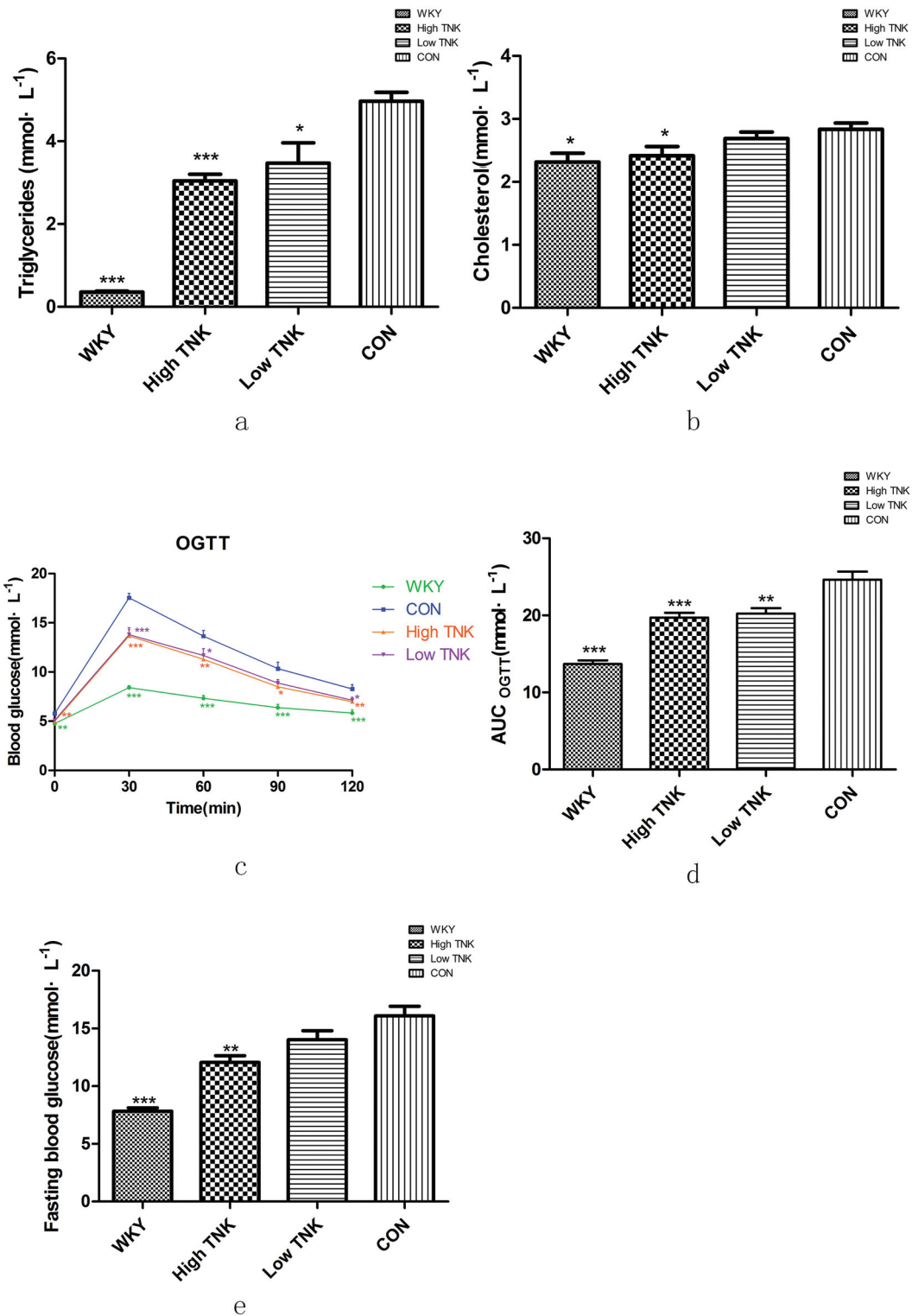


Figure 2. Blood lipid and blood glucose levels of rats in each group.

changes the microbial community of the obese rats, we investigate further to determine whether the metagenomes of gut microbiota would also be changed. By using a tool called PICRUSt, the metagenomes of each faecal sample were predicted according to 16S rDNA sequencing data. We then collapsed the predicted metagenome data into the KEGG pathway (level 3). A total of 104 pathways were changed in the CON group, while 8 pathways were changed in rats in the High TNK group (Figure 6).

Out of the eight pathways, six increased after administration of TNK formulation. The pathways that changed the most included atrazine degradation, pentose and glucuronate interconversions, riboflavin metabolism, thiamine metabolism, tropane, piperidine, and pyridine alkaloid biosynthesis, and tyrosine metabolism. These pathways were found at low levels in the CON group. Among the decreased pathways, the oxidative phosphorylation and RNA degradation pathways are at relatively high levels in the

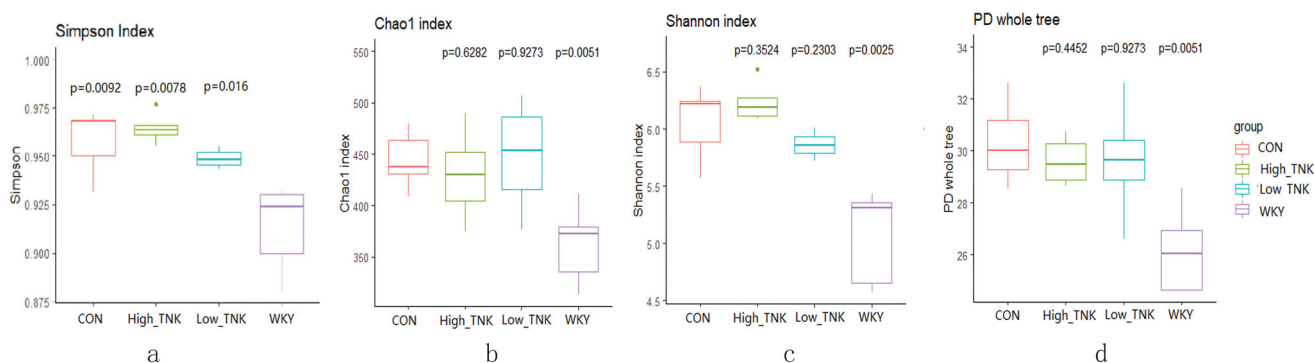


Figure 3. Significant changes between each group in alpha diversity index.

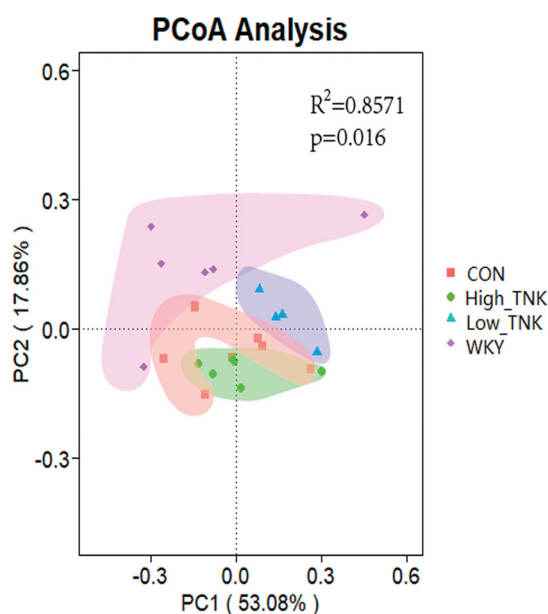


Figure 4. PCoA analysis in unweighted UniFrac distances based on OTU table of each sample.

CON group. Thus, these data suggest that metagenome changes play a role in the protective effect of TNK formulation.

## Discussion

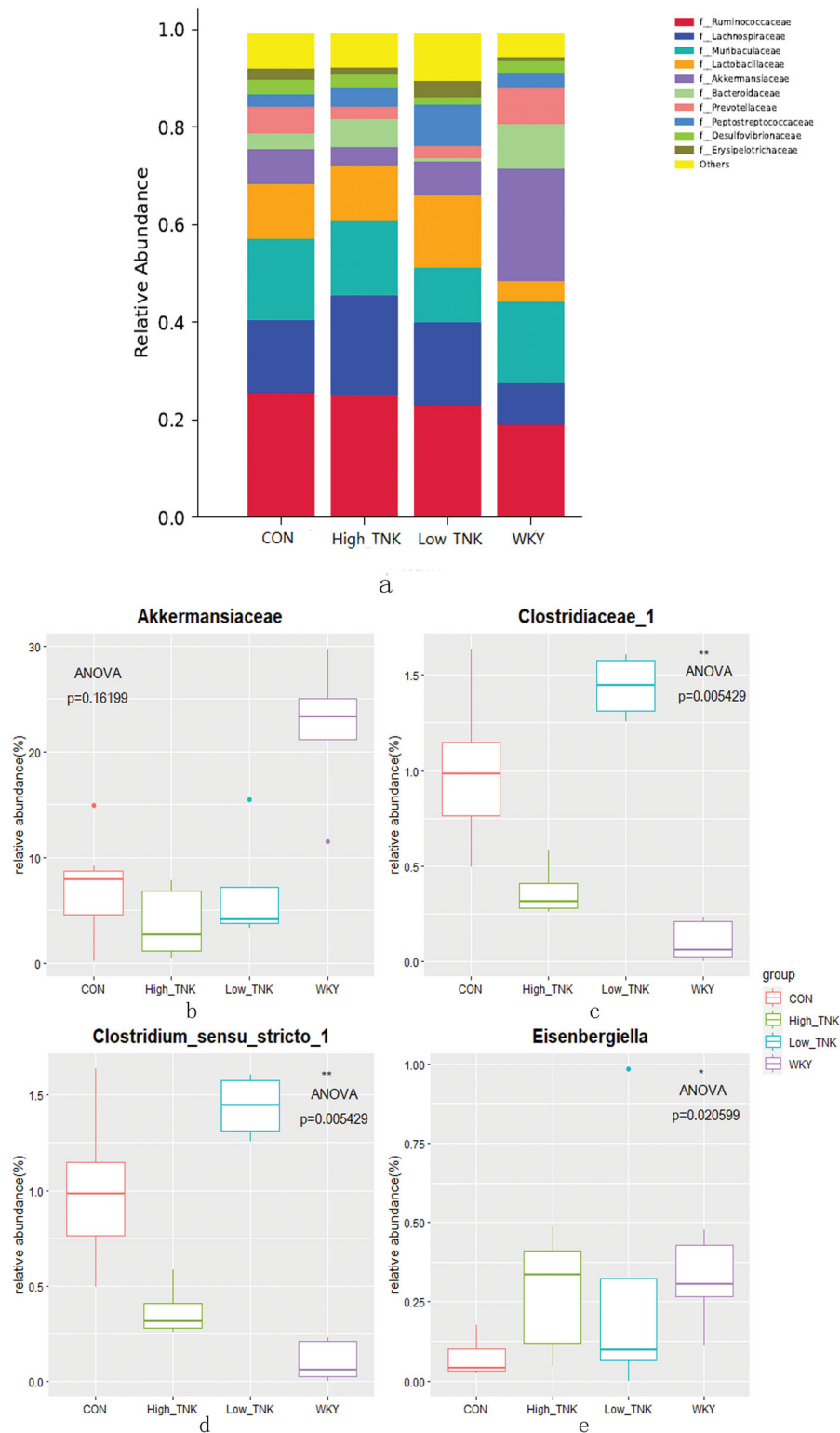
We demonstrated the role of TNK formulation in SHR/cp rats in modulating the composition of the gut microbiota, promoting the production of SCFAs, and alleviating the abnormalities in the metabolic pathways, including carbon fixation and energy and amino acid metabolism pathways, in obesity and other metabolic disorders. Hence, we, for the first time, provide evidence that the protective effect of TNK formulation on obesity and other metabolic disorders is most likely regulated by gut microbiota.

In 1963, Okamoto (Kyoto School of Medicine) used outbred Wistar Kyoto male rats with significant hypertension symptoms to mate with female rats with mild hypertension symptoms. Since then, brother-sister mating has been started and spontaneous hypertension traits have been continuously selected; In 1966, NIH introduced the 13th generation of the strain, named SHR/N. The SHR/NDmcr-cp (SHR/cp), which has a leptin receptor nonsense mutation (Tyr763→stop) in spontaneously hypertensive rats (SHR) has been characterised by obesity, hyperglycaemia, hyperlipidaemia, hyperinsulinemia, insulin resistance compared

with age-matched Wistar-Kyoto (WKY) rats. In the previous study, SHR/cp rats, T2DM and obesity model rats were used to study the effect of TNK formulation administration. Consistent with a previous study (Li et al. 2015), we found that TNK administration significantly reduced the plasma glucose levels and body weight in mice. Moreover, the low bioavailability of the chemical composition in TNK formulation had similar effects.

Different studies show that gut microbiota regulation could improve insulin resistance and body weight in metabolic syndrome. In particular, oral medications could regulate the gut microbiota directly. The effects of TNK formulation on gut microbiota regulation were evaluated by 16S rRNA gene sequencing. The study showed that *Eisenbergiella* spp. was remarkably increased while *Clostridium\_sensu\_stricto\_1* was notably decreased after the administration of the TNK formulation.

The genus *Eisenbergiella* (Lachnospiraceae) has been shown to be upregulated significantly in the TNK-formulation-treated rats. Its increase may affect health parameters (Plieskatt et al. 2013), such as infection (Bao et al. 2018) and functional dyspepsia (Luo et al. 2018). Also, this genus may be associated with diabetes in mice (Kameyama and Itoh 2014). Interestingly, it was reported that genera *Eisenbergiella* was significantly enriched in piglets from SF-fed sows and in suckling piglets from CON-fed sows (Cheng et al. 2018). Researchers (Amir et al. 2014) found that *Eisenbergiella* produces butyrate, acetate, lactate, and succinate as major metabolic products, which are important components of SCFA. SCFAs, mainly acetate, propionate, and butyrate, are produced by fermentation of undigested dietary proteins and fibres. SCFAs (Fan et al. 2017; Zhang et al. 2019) provided energy and nutrition to the intestinal epithelium, mitigate inflammation, and regulate glucose metabolism. SCFAs are important for health, and deficiency in SCFA production is associated with many diseases, such as T2DM (Zhao et al. 2018). Butyrate, in particular, is one of the preferred energy sources of the intestinal epithelium (Hamer et al. 2008). The increase in butyrate triggers intestinal gluconeogenesis, improving glucose and energy homeostasis and reducing hepatic glucose production, appetite, and body weight, along with increasing epithelial barrier function. It is reported that acetate has the ability to increase the risk of obesity by increasing the level of acetate in the ileum and colon by a gut-brain- $\beta$ -cell axis (Barranco 2016). In this study, the decreased abundance of butyrate-producing bacteria *Eisenbergiella* in the CON group might have hampered the production of butyrate, which probably led to the unprotected intestinal mucosa. However, the administration of oral TNK formulation alleviated the effects by increasing the genera *Eisenbergiella*. This is important for maintaining the integrity and the function of the gut barrier. This result indicates that TNK formulation may improve



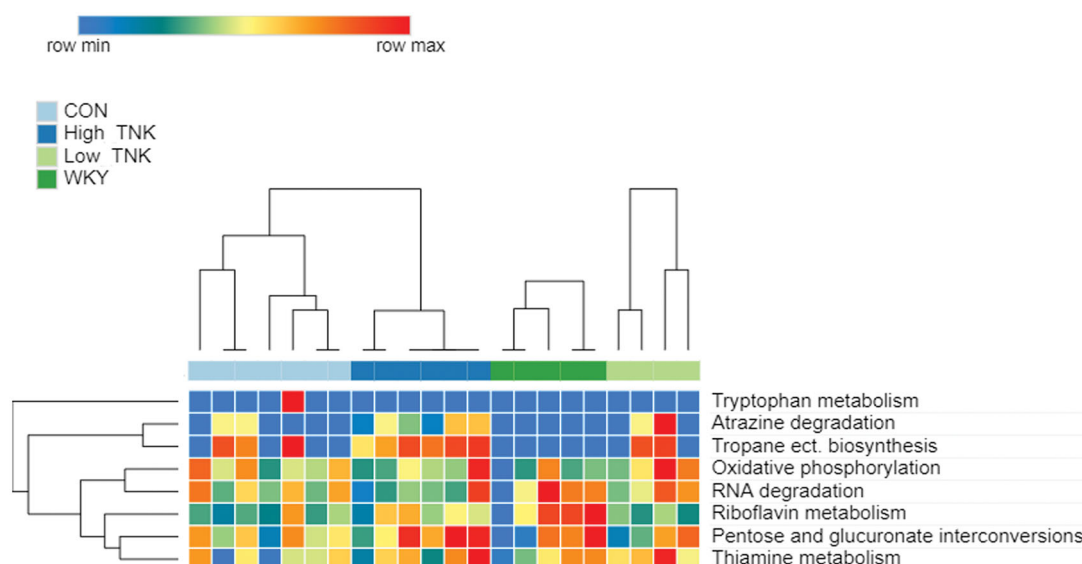
**Figure 5.** Changes of gut microbiota of rats in each group.

**Table 1.** Mann-Whitney test  $p$ -value was calculated between CON vs WKY and High TNK vs CON.

Tax	p-value (CON vs WKY)	p-value (High TNK vs CON)
Akkermansiaceae	0.000822106	0.161997526
Clostridiaceae_1	0.000961473	0.005429473
Clostridium_sensu_stricto_1	0.000961473	0.005429473
Eisenbergiella	0.001459794	0.0205987

the gut barrier function. However, more research is needed on the gut barrier.

*Clostridium\_sensu\_stricto\_1* spp. affects general metabolic health including Type 1 diabetes (Gao et al. 2019), inflammation (Xu et al. 2016), inflammatory bowel disease (Kiely et al. 2018), type 2 diabetes (Qin et al. 2017), and obesity (Neyrinck et al. 2016). It is the biomarker of previous GDM (Li et al. 2015) and plays an important role in nutrient digestion and fermentation.



**Figure 6.** PICRUSt2 pathway abundance heatmap with hierarchical clustering with Spearman rank correlation as metric.

In obese patients with metabolic abnormalities, *Clostridium\_sensu\_stricto\_1* is positively correlated with body weight (waist circumference and BMI) and blood lipid (low-density lipoprotein, TC and CHO), and becomes a biomarker for the classification of obesity-related metabolic abnormalities (Zeng et al. 2019). *Clostridium\_sensu\_stricto\_1* is also considered an opportunistic pathogen, which can proliferate and cause disease (Chang et al. 2014). Studies have shown that *Clostridium\_sensu\_stricto\_1* significantly increased, its abundance decreased after transplantation of healthy mouse flora, and it was significantly correlated with inflammation related genes (Wen et al. 2021). In this study, *Clostridium\_sensu\_stricto\_1* in obese diabetes rats (the CON group) was upregulated than normal rats (the WKY group), while the High TNK group was down-regulated. This may indicate that TNK can reduce the intestinal inflammation state by modulating *Clostridium\_sensu\_stricto\_1* and further affect the obesity indicators such as blood fat. However, the mechanism of its role needs to be further clarified.

The most increased pathways include atrazine degradation, pentose and glucuronate interconversions, riboflavin metabolism, thiamine metabolism, tropane, piperidine, and pyridine alkaloid biosynthesis, and tyrosine metabolism. These pathways are at relatively low levels in the CON group. In the decreased pathways, the oxidative phosphorylation and the RNA degradation pathways are at relatively high levels in the CON group. These data suggest that metagenome changes play an important role in the protective effect of TNK formulation. Furthermore, the altered microbiota revealed a strong correlation between the intermediate products and process of SCFAs and glucose metabolism. Likewise, pentose and glucuronate interconversions were related to the glucuronate pathway and the synthesis of fatty acids and sterols. Also, riboflavin and thiamine, known as vitamin B1 and B2, respectively, are the key enzymes for glucose metabolism. They are important for glucose metabolism, oxidative metabolism, and cell stress response. We found that energy metabolism was disturbed in the CON group rats. Our findings showed that TNK formulation might alleviate the metabolism disorders through changes in gut microbiota. However, the whole mechanism of upregulation and downregulation of the pathways needs to be clarified.

## Conclusions

This study proved for the first time that TNK preparation can reduce obesity. TNK regulates intestinal flora, up-regulates *Eisenbergiella*, and down-regulates *Clostridium\_sensu\_stricto\_1*, which is beneficial to the production of SCFAs, protects intestinal mucosa, reduces opportunistic pathogenic bacteria, and then regulates energy metabolism. It further provides support for the clinical application of TNK preparation, expands its indications, and provides guidance for obese patients.

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## Author contributions

Liu TH and Gao M conceived and designed the experiments. Tian P, Kudo M and Hayashi M performed the experiments. Tian P, Wu LL and Qin LL analysed the data. Tian P wrote the paper. Xu AL provided important help in the submission and revision of the manuscript.

## Disclosure statement

The authors declare that they have no conflicts of interest.

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