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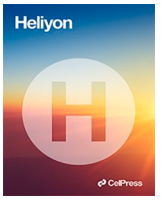

Research article

Exploring the effects of Tianma Gouteng granules on L-NAME-induced hypertensive rats based on 16S rDNA gene sequencing and metabolomics

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ABSTRACT

Background: A growing number of studies have shown that hypertension symptoms are closely related to intestinal flora. The body's metabolites are closely related to disease states. *Tianma* Gouteng Granules (TG), a traditional Chinese medicine compound, has been proven to be an effective compound for the treatment of hypertension by traditional Chinese medicine diagnosis, but the target and therapeutic mechanism of TG on hypertension are still unclear.

Aim of the study: We explored the mechanism of action of TG on hypertension by 16S rDNA gene sequencing and non-targeted metabolomics, verified the correlation between hypertension and intestinal flora, searched for potential markers of intestinal flora, and screened for the correlation between different flora and different metabolites, which facilitates a more scientific and reasonable guidance for the administration of TG.

Materials and methods: The hypertensive model rats were induced by L-NAME. After drug administration, 16S rDNA gene sequencing and non-targeted metabolomics were applied to detect and analyze the intestinal flora and fecal metabolites of the rats in each group. The Spearman coefficient method was used to construct the interactions system of different flora and metabolites, which explore the potential mechanism of TG treatment hypertension.

Results: After TG administration, the symptoms of hypertension were significantly reduced to normal in SD rats.16S rDNA gene sequencing and non-targeted metabolomics screened for differential flora *p_Actinobacteriota, o_Micrococcaceae, f_ Micrococcales, g_Rothias_Rothia_unclassified*, etc. and differential metabolites such as *L-Alanine* and *Hydroxyprolyl-Leucine*. TG treatment of hypertension was found to be associated with vitamin B6 metabolic pathway and lipid metabolic pathway.

Conclusions: TG can treat hypertension by affecting differential strains and differential metabolites, providing a scientific basis for guiding the rational use of TG.

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1. Introduction

Hypertension is an important risk factor for cardiovascular diseases and a major cause of premature death, which is a global public health problem [1]. According to the Global Health Observatory (GHO), 1.13 billion people in worldwide are affected by hypertension [2], and 245 million people in China had hypertension in 2021 [3]. Hypertension is the result of the superimposition of multiple factors, including genetic and environmental factors. And typical pathogenesis of hypertension includes overactivity of the sympathetic nervous system, activation of the renin-angiotensin-aldosterone system, vascular endothelial dysfunction, insulin resistance, and dysregulation of neurohumoral factors [4,5]. Currently, the main drugs commonly used in the treatment of hypertension are angiotensin-converting enzyme inhibitors, angiotensin II (Ang II) receptor antagonists, calcium channel blockers, β-blockers, and diuretics [6].

In recent years, intestinal microbiota and metabolomics analysis have received more and more attention in studying the mechanism of drug treatment of hypertension. A growing body of research has shown that imbalances in the gut microbiota play a key role in the development and progression of hypertension [7], and that hypertension can also significantly affect the structure and composition of the gut microbiota [8,9]. Metagenomics [40,41] has opened a new gateway for the exploration of microorganisms that are hitherto unknown. Specially, advancements in the metagenomics sequencing techniques have opened ways for the exploration and characterization of microorganisms that dwell in the human gut [42]. A recent study found that hypertension was associated with six bacterial genera and overall microbial richness [10]. A related study showed that some commensal bacteria produce ACE inhibitors, renin inhibitors, and antioxidant molecules during the digestion of mucin, which can trigger hypertension [11]. Some experiments have learned that probiotic fermented milk significantly reduces systolic and diastolic blood pressure in hypertensive patients [12], supplementation with Lactobacillus lactis reduces blood pressure in hypertensive mice [13,14]. In this study, we found that TG was able to lower blood pressure by restoring the imbalance of gut flora due to hypertension. In addition, hypertension can lead to changing in a variety of metabolites. Untargeted fecal metabolomics has been used to identify differential metabolites between drug-treated and control groups, which helps to screen biomarkers and study the biological processes involved in differential metabolites [15]. Therefore, studying the complex interactions between the hypertensive gut microbiota and metabolites provides new perspectives on the prevention, treatment and prognosis of hypertension.

Despite the continuous development of antihypertensive drugs, but can only control blood pressure and not a cure, traditional Chinese medicine in the treatment of hypertension means more abundant and effective. In Chinese medicine, hypertension belongs to the category of “vertigo”, “headache”, etc. Most of the patients are hypertensive with hyperactivity of liver and yang, and the pathogenesis of the disease is liver qi stagnation, liver fire, liver wind upturned. And the deficiency of kidney yin leads to the predominance of liver yang. So, the clinical treatment is based on calming the liver and submerging the yang, and tonifying the liver and kidneys. Clinical treatment is mostly based on calming the liver and submerging yang, tonifying the liver and kidney. TG is derived from Tianma Gouteng Drink in “New Meaning of Miscellaneous Diseases Certificate and Treatment”, which is a traditional Chinese medicine compound formula, clinically used in the treatment of hypertension, with the effect of calming the liver and extinguishing wind, clearing heat and tranquilizing the mind. It has obtained national patent (Patent No.: CN202130730788.2, Drug Lot No.: 20220806, Drug Record No.: Z51021084). The formula is as follows: 9 g of *Tianma(Gastrodia elata Bl.)*, 12 g of *Cyathula officinalis Kuan*, 12 g of *Crocus* sativus L, 18 g of *Haliotidis Concha*, *Gardenia jasminoides J. Ellis*, Eucommia ulmoides Oliv, *Scutellaria baicalensis Georgi*, *Leonurus japonicus Houtt*., *Taxillus chinensis*, *Telosma cordata (Burm. f.) Merr*, *Poria cocos(Schw.)Wolf*, are all 9 g. *Tianma* can act on the nerve center to regulate endothelin, angiotensin II and so on for decreasing blood pressure [16]. *Cyathula officinalis Kuan* polysaccharide have hypotensive effects [17]. *Crocus* sativus L is derived from the shells of Abelmoschus sinensis, and the aqueous extract of Cassia can inhibit angiotensin-converting enzyme (ACE) activity, with strong and long-lasting antihypertensive effects [18]. A large number of studies have found that all of these herbs have some degree of antihypertensive effect [19–24].

In the present study, 16s rDNA gene sequencing and metabolomics were used to study the relationship between intestinal flora and metabolic processes in L-NAME-induced hypertensive rats. Validated some of the mechanisms of action of TG in the treatment of hypertension and screened potential colony markers, metabolic markers and their pathways which will provide a reference for its further clinical studies.

2. Materials and methods

2.1. Experimental animals

27 SPF-grade male SD rats, 6–7 weeks old, body mass (190–210)g, provided by Hunan Slaughter Kingda Laboratory Animal Co. grade animal room with room temperature of 23 ± 2 °C, relative humidity of 50 ± 5 %, light exposure of 12 h/d, and free access to water. This study was conducted in strict compliance with the Guidelines for the Use and Management of Laboratory Animals issued by the National Institutes of Health (NIH) of the United States of America and the Implementing Rules for the Management of Medical Laboratory Animals issued by the National Planning Commission of the People’s Republic of China, and was approved by the Animal Ethics Committee of Wuhan Hualianke Biotech (HLK-20221115-001). 001) approved.

2.2. Materials, main reagents and instruments

Tianma Gouteng Granules (Batch No. 220806, Wuhan Hongshan Tongji Pharmacy); L-NAME (Shanghai Yuanye Biotechnology Co., Ltd., Batch No.); Captopril (Batch No. 220514, Shantou Jinshi Pharmaceutical Co., Ltd. of Sinopharm Group); methanol, acetonitrile

(LC-MS grade, CNW Technologies); ammonium acetate (LC-MS grade, SIGMA- ALDRICH); ammonia (LC-MS grade, Fisher Chemical); distilled water (Watson's); nitric oxide (NO, batch no. 20230324), purchased from Nanjing Jianjian Institute of Bioengineering; aldosterone (ALD, batch no. 20230310), angiotensin II (AngII, batch no. 20230310), endothelin (ET-1, batch no. 20230310) and endothelin (ET-1, batch no. 20230310). (ET-1, Lot No.: 20230310), Renin (Lot No.: 20230310), were purchased from Wuhan Beinlai Biotechnology Co.

Ultra-high performance liquid chromatography (Vanquish, Thermo Fisher Scientific); high-resolution mass spectrometry (Orbitrap Exploris 120, Thermo Fisher Scientific); centrifuge (Heraeus Fresco 17, Thermo Fisher Scientific); enzyme labeler (Multiskan FC, ThermoMultiskan FC); Centrifuge 5810R high-speed centrifuge (Eppendorf); electronic balance (BSA124S-CW, Sartorius, Germany); pure water system (Milli-Q, Millipore); ultrasonograph (Millipore, USA); and ultrasonic instrument (BSA124S-CW, Sartorius, Germany). Millipore); sonicator (PS-60AL, Shenzhen Redbond Electronics Co., Ltd.); Nova Seq6000 sequencer (Illumina); Phusion®High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs).

2.3. Methods

2.3.1. Animals and groups

After 1 week of acclimatization, the rats were divided into blank groups (C), model group (M) and tianma Gouteng granules group (TG), with 9 rats in each group. Hypertension model was established by administering ultrapure water supplemented with L-NAME 50 mg/(kg*d) [25] to SD rats for 2 consecutive weeks. The systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the rats were measured by using a noninvasive blood pressure measurement analyzer. The blood pressure of the rats was measured once a week, and the average value was taken from five consecutive blood pressure measurements each time. The hypertension model was successfully established if the systolic blood pressure (SBP) was >140 mmHg. Rats in the blank group (C) were given a normal diet and free access to water; The successful rat models (>140 mmHg) randomly divided into the model group (L-NAME 50 mg/kg) and the tianma Gouteng granules group (L-NAME 50 mg/kg + TG1.35 g/kg), were gavaged continuously for 3 weeks. One hour after the last administration, the feces of rats in each group were collected and placed in 2 m L dry and sterilized centrifuge tubes, and were stored in a refrigerator at −80 °C for testing. The feces of 6 rats were randomly taken from groups C, M and TG for 16S rDNA sequencing and metabolomics studies.

After experiment was finished, the rats in each group were fasted without food and water for 12 h before sampling, were anesthetized with 2 % pentobarbital sodium 0.2 mL/100 g intraperitoneally. Blood was collected from the abdominal aorta, and the abdominal aorta, heart, and liver were taken and weighed.

2.3.2. Morphological observations

Abdominal aorta was taken, fixed in 4 % paraformaldehyde, dehydrated, made transparent, embedded in paraffin, sectioned, stained with Hematoxylin Eosin (HE), and viewed under a microscope for morphological observation of the aorta.

2.3.3. ELISA

4 mL of blood was collected from the abdominal aorta and injected into an ordinary vacuum blood collection tube. After 30 min of rest, the blood was frozen and centrifuged at 3000 r/min for 15 min, and the serum was extracted. The levels of NO, ALD, AngII, ET-1 and Renin were detected by ELISA, and the experiments were performed strictly in accordance with the instructions of the kit, and the absorbance was measured at 450 nm.

2.3.4. Sequencing of 16S rDNA amplicons of rat intestinal flora

The metagenomic DNA of the fecal samples was extracted by SDS method, and the purity and concentration of the DNA were detected by agarose gel electrophoresis. The appropriate amount of DNA was taken in a centrifuge tube, and were diluted with sterile water to 1 ng/μL, which was used as a template for the amplification of the V3-V4 region of the 16S of the bacterial bacteria by using the specific primers with the Barcode and the high efficiency and high fidelity enzymes. The PCR products were purified and the library was constructed by using the library construction kit. The purified PCR products were evaluated using the library quantification kits of Agilent 2100 Bioanalyzer (Agilent, USA) and Illumina (Kapa Biosciences, Woburn, MA, USA) library quantification kits were evaluated. The library concentration of each up-sequencing kit will be qualified each up-sequencing library (Index sequences are not reproducible) after gradient dilution, mixed in the appropriate proportion according to the required sequencing volume, the sequenced libraries were mixed according to the required sequencing volume in the appropriate ratio and denatured by NaOH to single-stranded for on-line sequencing. The NovaSeq 6000 sequencer was used to sequence the double-end of the 2 × 250bp, and the corresponding reagents were NovaSeq 6000 sequencer. The reagent is NovaSeq 6000 SP Reagent Kit (500 cycles). At the same time, the abundance calculation of operational taxonomic unit (OTU), Alpha diversity and Beta diversity analysis were carried out, and T-test, LEfSe and other analytical methods were used to test the significance of differences in the species composition of the samples, and to excavate the species with significant differences among the groups.

2.3.5. Fecal untargeted metabolomics in rats

Weigh 25 mg of sample, add 500 μL of extraction solution (methanol: acetonitrile: water = 2:2:1 (V/V), containing isotope-labeled internal standard mixture), grind the process for 4 min at 35 Hz, sonicate for 5 min (ice-water bath), and after repeating the steps for three times, −40 °C static for 1 h, the sample will be 4 °C, 12,000 rpm (centrifugation force of 13,800 (× g), radius of 8.6 cm) The supernatant was centrifuged for 15 min. The separation was performed on a Waters ACQUITY UPLC BEH Amide (2.1 mm × 50 mm,

1.7 μm) liquid chromatography column using Vanquish (Thermo Fisher Scientific) ultra performance liquid chromatography (UPLC). Then the metabolites were analyzed by mass spectrometry on an Orbitrap Exploris 120 mass spectrometer to identify the metabolites, and the primary and secondary spectra of the quality control (QC) samples were collected to identify the metabolites. The data were pre-processed by ProteoWizard, and then analyzed by uni-dimensional and multi-dimensional statistical analysis, and the volcano plots were drawn by R software.

2.3.6. Joint analysis of the correlation between rat gut flora and metabolites

Spearman's correlation analysis was performed on the significantly different colonies at each level of 16S rDNA sequencing and the significantly different metabolites in metabolomics to mine the colony-metabolite interactions.

2.4. Statistical methods

Comparisons between groups were analyzed by one-way ANOVA using SPSS 23.0 software, and data were presented in mean ± SD. OPLS-DA VIP>1.0 and $p < 0.05$ were used as screening criteria for metabolites with significant differences. Correlations between differential gut flora and differential metabolite data were calculated using the Spearman algorithm.

3. Results

3.1. Effect of TG on clinical symptoms in L-NAME-induced hypertensive rats

As shown in Fig. 1a, after 1–2 weeks of drug administration, SBP and DBP of rats were significantly increased by L-NAME in group C compared with group M ($P < 0.01$,$P < 0.05$) (an increase of >20 mm Hg and both of them were >140 mm Hg). After three weeks of drug administration, SBP and DBP of TG group were significantly decreased compared with that of the model group ($P < 0.01$), and the SBP of the TG group was <140 mmHg. Hypertension had a significant effect on the liver and heart coefficients of rats in group C compared to group M ($P < 0.05$). The organ coefficients of the TG group gradually converged to the rats of the C group after the administration of the drug (Fig. 1b). As shown in Fig. 1d, the intima of abdominal aorta of group C rats was intact, the thickness of the middle membrane was moderate, the muscle fibers were arranged in an orderly manner, and the proliferation of vascular smooth muscle cells was not seen, and the level was clear; whereas the intima of rats in group M was thickened, and the structure was damaged and disorganized, and the intima of the vessels in group M group tended to be smooth, the structure was tightened, and the degree of the reconstruction of vascular wall was reduced after the treatment of the drug. In conclusion, L-NAME successfully induced hypertensive rat model, and TG administration improved the related symptoms.

3.2. ELISA results

From Fig. 1c, it can be observed that compared to Group C, the concentrations of ALD, Ang II, ET-1, and Renin in the serum of rats in Group M significantly increased ($P < 0.01$), while the concentration of NO significantly decreased ($P < 0.01$). In comparison to Group M, the concentration of NO in the serum of rats in Group TG significantly increased ($P < 0.01$), while the concentrations of ALD, Ang II, ET-1, and Renin significantly decreased ($P < 0.01$). The results from the cytokine analysis indicate that after administration of TG, the symptoms of hypertension in rats can be significantly improved.

3.3. Differential analysis of rat intestinal flora

3.3.1. Alpha and beta diversity analysis of rat intestinal flora in each group

The dilution curves of all samples flattened, which indicated that the sampling volume of this experiment is reasonable and the species composition is rich (Fig. 1e). Alpha indicates the diversity of species within the samples, mainly including Chao1, Shannon and Simpson indices. As shown in Fig. 1g, there is no significant difference between C, M and TG groups on Chao1, Shannon and Simpson indices, but C and TG are more similar on Chao1, Shannon and Simpson. Beta denotes the diversity of species between samples were summarized by principal co-ordinates analysis (PCoA) (Fig. 1f), which showed that the composition and structure of the intestinal flora of rats in group M changed significantly compared with that of rats in group C. The intestinal flora of rats in group TG deviated from group M close to group C, indicating that TG had the effect of regulating the structure of the intestinal flora of rats with hypertension and attenuating hypertension-induced bacterial dysbiosis. In conclusion, TG could regulate the diversity of rat intestinal flora, and although different groups were similar in species richness, there were still a few differences in the species of flora.

3.3.2. Species composition and difference analysis of rat intestinal flora in each group

From Fig. 2a and c, it can be observed that at the phylum level, TG administration can regulate the relative abundance of *Firmicutes* and *Firmicutes/Bacteroidetes*(F/B)ratio in hypertensive rats. The F/B ratio is an important parameter reflecting the disorder of intestinal flora, and a high F/B ratio is commonly considered an indicator of hypertension. *Actinobacteriota* is closely related to the homeostasis of gut microbiota. In this experiment, the F/B ratio in hypertensive rats significantly decreased after TG administration ($P < 0.05$), and the relative abundance of *Actinobacteriota* significantly increased after TG administration ($P < 0.05$). From Fig. 2b and d, it can be seen that at the genus level, the TG group could increase the relative abundance of *Akkermansia* and *Lactobacillus* in hypertensive rats, while decreasing the relative abundance of *Clostridia_Ucg-014_unclassified*. Overall, it can be concluded from the phylum and genus levels that

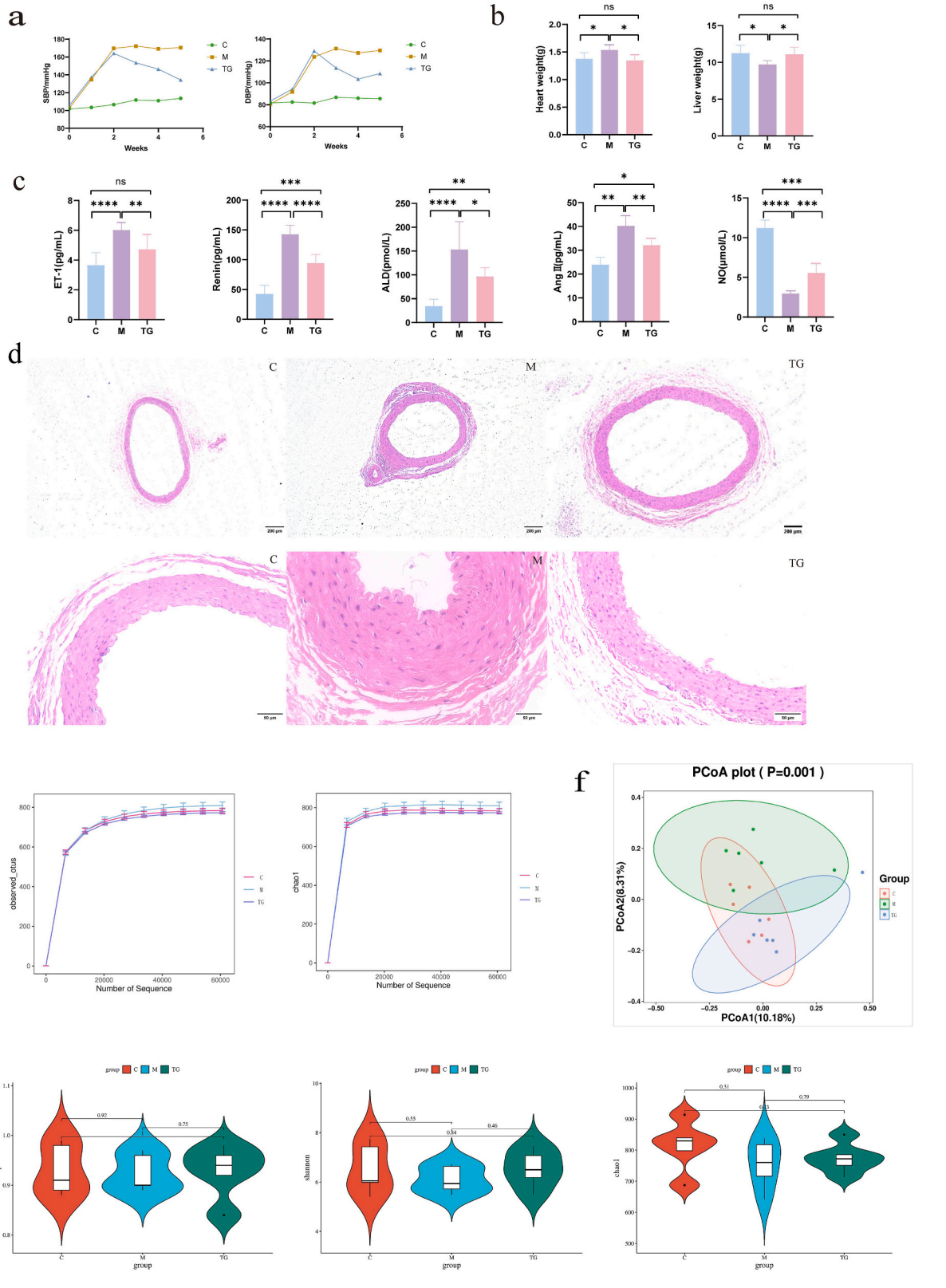

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Fig. 1. TG was able to improve L-NAME-induced hypertension symptoms in rats. a. Periodic changes of SBP and DBP in rats of C, M and TG groups. b. Organ index in rats of C, M and TG groups. c. Serum concentrations of ALD, AngII, ET-1, Renin NO concentrations in serum of rats in C, M and TG groups. d. HE staining micrographs of abdominal aorta in rats in C, M and TG groups; e. Sample dilution curves of otus. f. Principal co- ordinates analysis, PCoA, of rats in C, M and TG groups. g. Chao1, Shannon and Simpson indices of rat intestinal flora in C, M and TG groups. *$P < 0.05$, **$P < 0.01$,***$P < 0.001$,****$P < 0.0001$ ($n = 6$).

the TG group can regulate the relative abundance of gut microbiota in hypertensive rats, thereby achieving the goal of lowering blood pressure.

3.3.3. Screening of key differential flora between groups of rats in each group

Significant differences in species between groups were identified using LEfSe analysis (LDA >2.5, $P < 0.05$). Fig. 2e-f illustrates that there were 30 distinct species between groups C and M, while 8 unique species were found between groups M and TG. Specifically, 5 key different species exhibited significant variances among groups C, M, and TG, namely *p_Actinobacteriota*, *o_Micrococcales*, *f_Micrococcaceae*, *s_Rothia_unclassified*, and *g_Rothia*. See Fig. 2g for details. In conclusion, following treatment with TG, hypertensive rats demonstrated the ability to regulate critical species in the gut microbiota, thereby ameliorating the symptoms of hypertension.

3.4. Fecal metabolomics analysis of hypertensive rats treated with Tianma Gouteng granules

3.4.1. Statistical analysis of fecal metabolites in rats

Based on the metabolomics data collected by UHPLC-OE-MS in positive-negative ion mode, principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) models were established to obtain the PCA score plots (Fig. 3a-d) and OPLS-DA score plots (Fig. 3e–f). The results of the PCA showed that the C group could be completely distinguished from the M group in the positive and negative ion modes, which showed that the metabolic state of the body of hypertensive rats was significantly altered. The TG group was completely distinguished from the M group after drug administration. Further analysis by using OPLS-DA showed that the scatters of the C group and the M group could be significantly distinguished, which verified the results of the PCA analysis and indicated that the modeling of the hypertensive rat model had been established successful. R2X, R2Y, and Q2 were the important predictive parameters of the OPLS-DA model. The model is stable as R2Y and Q2 closed to 1. In general, a valid model is $Q2 > 0.5$ and an excellent model is $Q2 > 0.9$ (Miao et al., 2021). The results in this study showed that the OPLS-DA model validated the positive ion model with $R2 = 0.88$ and $Q2 = 0.89$, which suggested the model stable and reliable. Volcano plots were formed based on the combined application of fold change (FC) analysis and *t*-test to analyze the significance of metabolite changes between the two samples.

3.4.2. Analysis of differential metabolites in rat feces

Metabolomics results showed that hypertension altered fecal metabolites in rats. Differential metabolites were obtained based on $FC > 1.0$, *t*-test $P < 0.05$, and VIP value > 1 in OPLS-DA model. A total of 73 metabolites were selected as marker metabolites for hierarchical clustering analysis (up-regulated or down-regulated in the M group compared to the C group, down-regulated or up-regulated in the TG group compared to the M group (see Fig. 4a). There were 67 significantly different metabolites between the M and TG groups in the positive ion mode, and 6 significantly different metabolites were identified in the negative ion mode, including *L-alanine*, *Phenylalanyl-Alanine*,*4-Hydroxybenzoic acid, γ-glutamylmethionine*, *2,3-Deoxyerythronic acid*, *4-Hydroxyvalsartan and 3-Nitrotyrosine, etc*. The main metabolites were found to be related to amino acid metabolism, and after TG administration, the fecal metabolites of the TG group rats returned to the level of the C group rats. In conclusion, the metabolomics results revealed that TG does indeed improve the symptoms of hypertensive rats, and its metabolic pathways are likely closely related to amino acid metabolism. However, the exact mechanism still requires further research.

3.4.3. Analysis of related metabolic pathways

Enrichment analysis of the KEGG pathways, as shown in Fig. 4b, revealed that the metabolic pathways with the most significant effects were vitamin β-alanine metabolism, pantothenate and CoA biosynthesis, steroid hormone biosynthesis, arginine and proline metabolism, B6 metabolism and B6 biosynthesis, Pyrimidine metabolism, Glycerophospholipid metabolism, and lipid metabolism. Glycerophospholipid metabolism.

3.5. Combined analysis of differential flora and differential metabolites in rats

In order to assess the interaction between differential gut flora and differential fecal metabolites, Spearman correlation analysis was performed between the five key differential flora screened for TG in 3.3.3 and the 20 marker metabolites screened in 3.4.2 (Specific data can be found in (Table S1). As shown in Fig. 4c correlation clustering heatmap showed that *O_Micrococcales* was significantly negatively correlated with *Uracil*, and *L-Alanine* as significantly correlated with *p_Actinobacteriota*, *o_Micrococcales*, *f_Micrococcaceae*, *s_Rothia_ unclassified*, and *g_Rothia* were significantly positively correlated, and *Hydroxyprolyl-Leucine* was positively correlated with *p_Actinobacteriota*, *o_Micrococcales*, *f_Micrococcaceae*, *s_Rothia_ unclassified*, and *g_Rothia* were significantly negatively correlated.

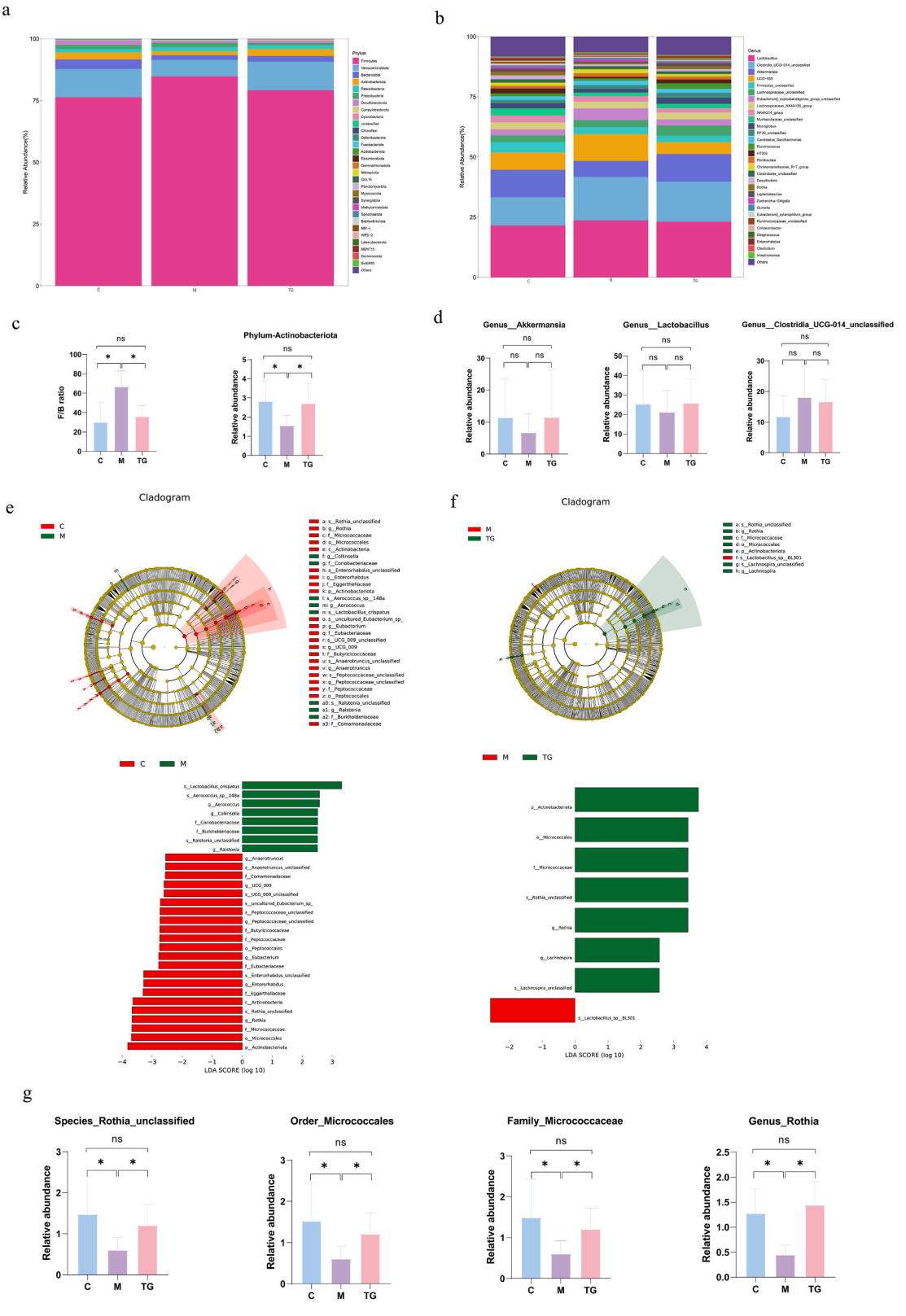

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Fig. 2. TG regulates the composition of hypertensive intestinal flora restoring the imbalance of intestinal flora. a. Species and content of the flora of C, M and TG groups presented at the phylum level. b. Species and content of the flora of C, M and TG groups presented at the genus level. c. Significant differences between C, M and TG groups on F/B and *Actinobacteriota*. d. Differences between C, M and TG groups on *Akkermansia*, *Lactobacillus* and *Clostridia_Ucg-014_unclassified*. e-f. LEfSe analyses of C, M and TG groups. g. Significantly differentiated bacterial groups in the LEFSe analyses *Species_ Rothia_unclassified*, *Order_Micrococcales*, *Family_Micrococcaceae* and *Genus_Rothia*.*$P < 0.05$, **$P < 0.01$,***$P < 0.001$,****$P < 0.0001$ ($n = 6$).

4. Discussion

This study confirmed the efficacy of TG in the treatment of hypertension. This experiment elucidated some of the pharmacological effects of TG in the treatment of hypertension. The targets and pathways of action of TG for hypertension were also predicted. Firstly, it was concluded from the results of tail blood pressure measurement in SD rats that TG could significantly reduce DBP and SBP in hypertensive SD rats after two weeks of TG administration. Then it was found from the results of organ index and abdominal aorta section that the organ index of TG group was significantly close to normal group. The thickness of the mesenteric layer of the vessel wall of the abdominal aorta showed a decreasing trend after TG administration, and the connective tissue of the vessels was clearly and neatly arranged after the administration of TG. Finally, we found that Renin, ALD, ET-1, and AngII, which are blood pressure indicators, were significantly decreased after TG administration, while NO, which is a blood pressure indicator, was significantly increased after TG treatment. In conclusion, it was found that TG has a very good effect on blood pressure lowering through the physiological indexes of rats, and it has a good development prospect in the market of antihypertensive drugs in the future.

Chao1, Shannon and Simpson indices were obtained by 16S rDNA sequencing analysis, which presented that the alpha diversity of the intestinal microbiota of rats in the model group was elevated, and the increase of harmful flora in intestinal microorganisms affects the intestinal function and induces various diseases [26]. After TG treatment, the harmful flora decreased, the microbial diversity decreased, and intestinal flora disorders were restored. In this study, PCoA analysis was used to analyze the similarity (β-diversity) of the intestinal microbiota of the samples. The PCoA plot revealed clear clustering profiles between the samples of each group, and the beta diversity of rats in the hypertensive group was significantly different from that of the C and TG groups. These results suggest that TG can indeed treat hypertension by regulating the intestinal flora.

In this study, we found that TG could have a hypotensive effect by modulating the composition of rat intestinal flora. At the portal level, it regulated the abundance of *Thick-walled Bacteroidetes*, *Bacteroidetes* and *Warty Microbacteroidetes*. An elevated F/B ratio is often used as a marker for evaluating the imbalance of intestinal flora [27]. The imbalance of the intestinal flora of hypertension was mainly characterized by high abundance of *Thick-walled Bacteroidetes* and low abundance of *Bacteroidetes*, and the elevation of the F/B values [28]. The F/B values of the intestinal flora of rats in the TG group were significantly lower compared to the model group. The results of this experiment suggest that TG is able to regulate the imbalance of intestinal flora, which leads to the treatment of hypertension. TG accelerates the production of acetic and butyric acids in the *phylum Mycobacterium*. In previous study, it is known that acetic acid can reduce plasma renin activity and aldosterone level in rats, which is related to vasoconstriction [29]; butyric acid can be involved in the regulation of cardiovascular centers through vagal afferent fibers, thus regulating blood pressure [30]. In addition, *P_Actinobacteriota* was the dominant flora in the control group, and the relative abundance of *P_Actinobacteriota* was significantly higher in the TG group compared with the model group ($P < 0.05$). At the genus level, TG up-regulates the relative abundance of *G_Akkermansia* and *G_Lactobacillus* and down-regulates the relative abundance of *G_Clostridia_UCG-014_unclassified*. Numerous studies [31] have shown that the lack or decreased abundance of this commensal bacterium was linked with multiple diseases (such as obesity, diabetes, liver steatosis, inflammation and response to cancer immunotherapies). Previous studies have shown that *Akkermansia* was found to decrease in both hypertensive patients and rat models of hypertension [32], which is consistent with the results of this study. *Genus_Lactobacillus* belongs to the probiotic genus of intestinal flora. The angiotensin I inhibitory peptide released during its fermentation process reduces the conversion of angiotensin I to angiotensin II, thus lowering the blood pressure effect [33]. Thus, in the present experiment, it was found that TG treatment of hypertensive rats affects the abundance of relevant strains of bacteria in the intestinal flora of the rats. Similarly, the differential strains of the intestinal flora in turn affect the development of the hypertensive disease process through intestinal metabolism.

Intestinal flora can metabolize almost all essential amino acids, and amino acid catabolism plays a key role in regulating the intestinal barrier and immune response [34,35]. The results showed that *L-Alanine* was positively correlated with *p_Actinobacteriota*, *f_Micrococcaceae*, *o_Micrococcales*, and *g_Rothia*, etc. *L-Alanine* is a metabolic marker for blood pressure and risk of cardiovascular disease [36], *Alanine* levels were decreased in both patients with hypertension and group M rat models, which is consistent with the results of the present study. Related studies have shown that *Phenylalanyl-Alanine* is negatively correlated with hypertension [37]. And *Phenylalanyl-Alanine* can interfere with the production of *tetrahydrobiopterin* (BH4), a cofactor for the hydroxylation of aromatic amino acids, which is involved in the relaxation of the endothelium [38]. The oxidation of BH4 at low levels of *Phenylalanyl-Alanine* may lead to an alteration of its vasoactive alteration of properties and deleterious effects on endothelial cells, thus causing hypertension [39]. The *Phenylalanyl-Alanine* level in group M was significantly lower than that in the normal group, and the *Phenylalanyl-Alanine* level was rebounded after treatment with TG. In summary, TG plays a therapeutic role in hypertension mainly by regulating alanine *L-Alanine* metabolism and *Phenylalanyl-Alanine* metabolism.

Of course, there are still some shortcomings in this experiment. Firstly, TG is a TCM prescription, which consists of eleven kinds of herbs, so it is difficult to characterise the composition of TG compound, and some chemical-physical reactions may occur between the single components of different herbs. In addition, there was no positive drug group in this pharmacological experiment, so there was no

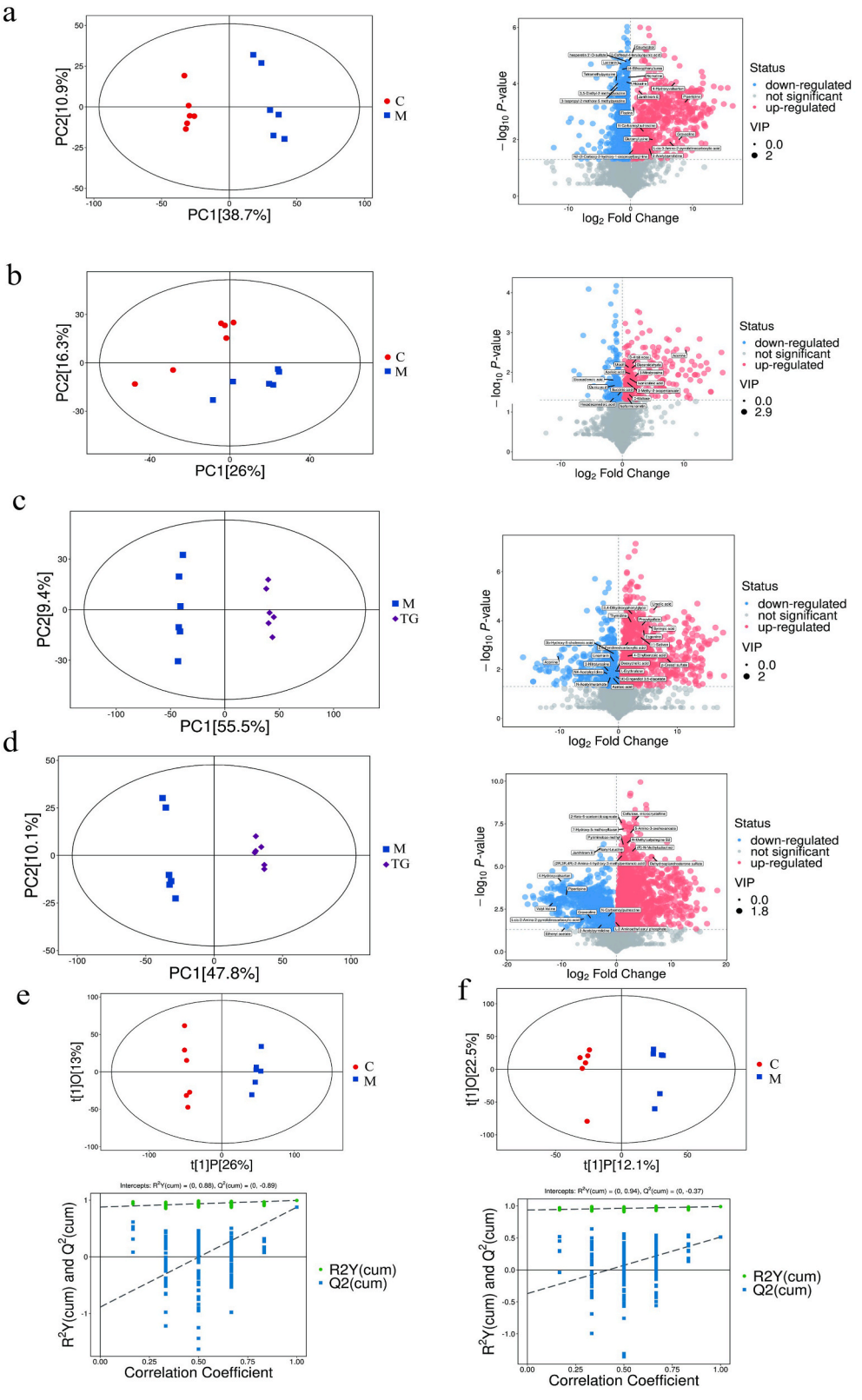

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Fig. 3. a. Plot of PCA scores between groups C and M in positive ion mode and volcano plots of differential metabolites between the two samples; b. Plot of PCA scores between groups C and M in negative ion mode and volcano plots of differential metabolites between the two samples; c. Plot of PCA scores between groups M and TG in positive ion mode and volcano plots of differential metabolites between the two samples; d. Plot of PCA scores between groups M and TG in negative ion mode; e-f. Plot of OPLS-DA scores between groups C and M in positive and negative ion modes. PCA score plots and differential metabolite volcano plots between the two groups of samples; e-f. OPLS-DA score plots between groups C and M in positive and negative ion mode.

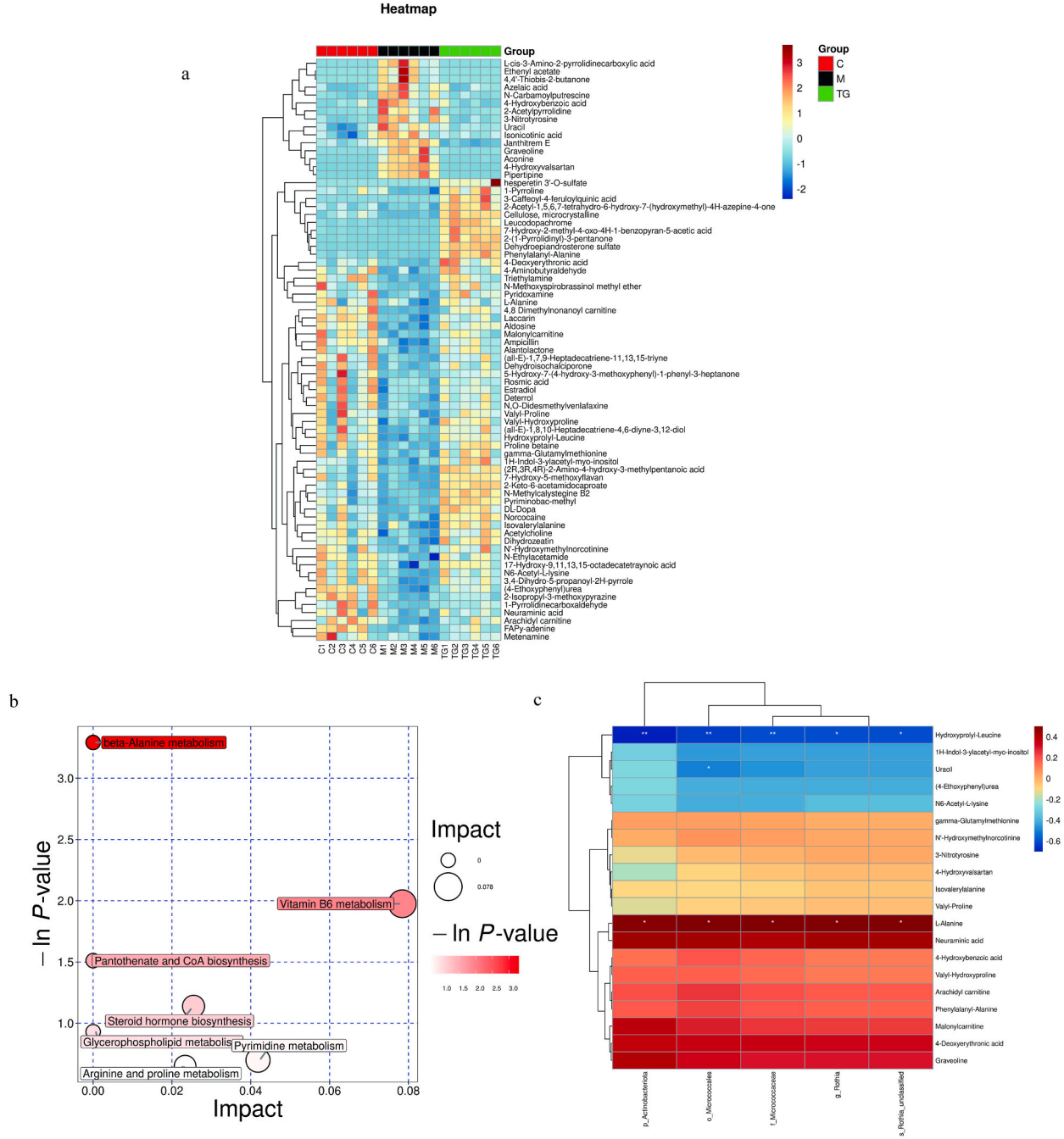

Fig. 4. a. Heat map of 73 differential metabolites in C, M and TG groups; b. KEGG pathway enrichment analysis of differential metabolites in M and TG groups; c. Correlation analysis of 5 differential flora and 20 differential metabolites.

comparative benchmark for the efficacy of TG. This affects the credibility of the experimental results. Furthermore, although the differential strains affecting hypertension were screened in this experiment, the validation experiment of faecal transplantation was not done, so the subsequent experiments need to be further improved. Finally, metabolomics prediction of metabolic pathways such as amino acids needs to be further validated using western blotting. For the above problems, we will solve them one by one in the follow-up experiments.

5. Conclusion

In summary, this study preliminarily confirmed that TG has an ameliorative effect on L-NAME-induced hypertension, which is related to increasing the diversity of intestinal flora, maintaining intestinal micro ecological balance, and regulating amino acid metabolism disorders. This conclusion provides a certain scientific basis for the clinical application of TG in the treatment of hypertension and lays the foundation for further elucidation of its potential mechanism.

CRediT authorship contribution statement

Li Cheng: Writing – review & editing, Writing – original draft, Data curation. **Zhenyang Huang:** Writing – original draft, Software, Investigation, Funding acquisition. **Jiawei He:** Software. **Xinyi Zhang:** Project administration. **Jiangxue Di:** Software, Resources, Methodology, Investigation. **Hanmei Jiang:** Validation, Software, Resources, Funding acquisition. **Yi Liu:** Writing – review & editing, Writing – original draft, Resources.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

Ethics statement

This study was conducted in strict compliance with the Guidelines for the Use and Management of Laboratory Animals issued by the National Institutes of Health (NIH) of the United States of America and the Implementing Rules for the Management of Medical Laboratory Animals issued by the National Planning Commission of the People's Republic of China, and was approved by the Animal Ethics Committee of Wuhan Hualianke Biotech (HLK-20221115-001). 001) approved. All participants provided written informed consent to participate in the study and for their data to be published.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e41786.

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Abbreviations

TG: Tianma Gouteng Granule
GHO: Global Health Observatory
Ang II: Angiotensin II
ACE: Angiotensin-converting enzyme
ox-LDL: Oxidized low density lipoprotein
ACE2: Angiotensin-converting enzyme 2

NIH: National Institutes of Health
SBP: Systolic blood pressure
DBP: Diastolic blood pressure
HE: Hematoxylin Eosin
OTU: Operational taxonomic unit
UPLC: Ultra performance liquid chromatography
F/B: Firmicutes/Bacteroidetes
PCA: Principal component analysis
OPLS-DA: Orthogonal partial least squares discriminant analysis