Research article

Fufang Duzheng tablet attenuates adjuvant rheumatoid arthritis by inhibiting arthritis inflammation and gut microbiota disturbance in rats

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ARTICLE INFO

ABSTRACT

Objective: To explore the treatment effect and potential mechanism on gut microbiota, nutrition, and metabolism of Fufang Duzheng Tablet (DZGP) on rheumatoid arthritis (RA).
Methods: Collagen-induced arthritis rats' models were established and divided into three groups: model control group (FK), DZGP group (FZ, 0.45 g/kg/d), and methotrexate group (FM, 1.35 mg/kg), which were treated by gavage for 28 days. The physiopathologic changes of joints and body weight in each group were recorded; the morphology of synovial and ankle tissues was observed by hematoxylin-eosin staining, and the level of serum TNF-α and IL-1β was tested by ELISA. UPLC/MS-MS and network pharmacological analysis were used to identify the serum components, and 16S rDNA sequencing analysis was applied to the intestinal contents of rats.
Results: DZGP treatment significantly alleviated arthritis symptoms, pathological manifestations, toe thickness, and TNF-α and IL-1β levels in RA rats. We identified 105 metabolites and 18 components in the serum of DZGP-group rats. The main therapeutic targets of DZGP for anti-RA were TP53, epidermal growth factor receptor, and AKT1. Molecular docking showed that there was good binding efficiency between core components and main targets. 16S rDNA sequencing showed that DZGP treatment regulated the structure of the gut microbiota.
Conclusion: DZGP showed a good anti-inflammatory effect on RA and played an important role in improving the structure of the gut microbiota in RA rats.

1. Introduction

Rheumatoid arthritis (RA) is a chronic disease characterized by significant clinical symptoms such as synovial hyperplasia and chronic arthritis. These symptoms can lead to irreversible joint damage and significant disability [1]. Each year, more than 1 % of the

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global population suffers from RA [2]. Its etiology and pathogenesis remain unclear. Firstly, joint swelling in RA is indicative of inflammation in the synovial membrane, a consequence of immune activation. and is correlated with macrophages, fibroblast-like synoviocytes, as well as inflammatory cytokines such as interleukin IL-6, IL-1β, and TNF-αinflammatory mediators [3]. Secondly, RA is considered an autoimmune disease accompanied by metabolic alterations, which fulfill bio-energetic requirements, promote cell proliferation, drive inflammatory mediator secretion, mediate leukocyte infiltration, induce joint destruction and muscle atrophy, and regulate cell proliferation [4]. Lastly, microbial infection is deemed a significant inducer of RA. Alterations in the composition of intestinal bacteria in individuals with preclinical and established RA suggest a vital role of the gut microbiota in the immune dysfunction characteristic of RA [5].

Currently, there is no effective treatment for RA. There are several main types of drugs for RA, including biological, and new non-biological disease-modifying antirheumatic drugs and Chinese medicines, which lead to numerous side effects after long-term use [6]. Sustained remission and maintenance of low disease activity are goals in the management of this disease [7]. Accordingly, new therapies are urgently needed by RA patients.

Duzhenggan, also known as *Vitis amurensis,* is a variety of *Ampelopsis brevipedunculata* (Maxim) Traut. As per traditional Chinese medicine literature, it is primarily used as the main component of a plaster for promoting bone healing, increasing vascular permeability, and resisting inflammation and detumescence. Due to its excellent clinical therapeutic effects, it was later processed into oral tablets by local hospitals and named Fufang Duzheng Tablet (DZGP) [8,9]. DZGP is a drug developed by Enshi Central Hospital, originating from the folk prescription of the Tujia nationality in Enshi, Hubei Province. It comprises three Tujia medicines: Duzhenggan, Acanthopanax Senticosus, and Cilaobao [10]. Duzhenggan, a Tujia national medicine, originates from the roots and leaves of *Ampelopsisbo Dinieri* (Levi. Etvant) Rehd. of Vitaceae in Badong County. It is spicy, sweet, and cool in nature, and is known for promoting blood circulation, removing blood stasis, reducing swelling, and relieving pain [11,12]. Acanthopanax senticosus, a dicotyledoneae of Acanthopanax and Araliaceae of Araliaceae, has a pungent taste and a warm nature. It can dispel wind and dampness, nourish the liver and kidney, and strengthen bones and muscles [13]. *Araliachinensisl. a. Echinocaulishand. mazz*, the root bark, root, stem bark, and leaves of *Aralia elata* or *Aralia elata* of Araliaceae, are flat in nature. They have the functions of dispelling wind and removing dampness, removing blood stasis and swelling, stopping bleeding, and relieving pain [14]. The combination of these three drugs promotes blood circulation, expels wind and dampness, relieves pain, and reduces swelling [15]. Over more than 30 years of experimental research and clinical practice, the bone-setting ointment has matured and can be used for various diseases such as fractures, tendon injuries, arthritis, endocrine diseases, and more [16–18]. In the past, treatment and research were primarily used for external applications to treat arthritis. Fufang Duzheng Tablet was made into an internal medicine based on three drugs of compound Jiegu Plaster, which was used to treat gout, arthritis, and other diseases [19–21]. Most of the existing literature reports are clinical summaries, and there is no in-depth study on the therapeutic mechanism of this drug for RA.

In the Enshi area, Duzhenggan Jiegu ointment is widely used as an anti-RA drug, which is further modulated by Enshi Central Hospital. Ultimately, it's called DZGP. The Acanthopanax senticosus can regulate the gut microbiota and improve the intestinal environment in Parkinson's disease, and the extract can significantly increase the abundance of *Bifidobacterium longum*, thus inhibiting focal cerebral ischemia [22–25]. In RA patients, the intestine of gram-negative bacteria decreased but gram-positive bacteria increased. What's more, Prevotellaceael, Collinsella, and Eggert Hella were often found to increase, and the diversity was signally raised [26,27]. Most importantly, an unbalanced gut microbiota can be reversed after drug treatment [28]. Therefore, RA may benefit from therapeutic strategies that regulate the gut microbiota. Recently, numerous studies have shown that the gut microbiota in in control of traditional Chinese medicine [29–31]. According to the above research results, it is very likely that DZGP will play a role in treating RA by affecting the gut microbiota. However, the protective mechanism of DZGP on RA remains unclear.

In this study, we use collagen-induced rat models to explore the effect of DZGP on RA, focusing on plasma metabolomics, network pharmacology, and 16S rDNA sequencing of gut microbiota changes, revealing a comprehensive and reliable relationship between gut microbiota and human health, nutrition, and metabolism after treating RA with DZGP.

2. Materials and methods

2.1. Reagents and materials

DZGP were purchased from the pharmacy of Enshi Central Hospital (Hubei, China), lot number: 2022040, 2016055, size: 0.36 g/tablet. Methotrexate (analytical grade) was purchased from Sine Pharmaceutical Laboratories Company (Shanghai, China). HE staining-related reagents and ELISA kits used in the experiment were purchased from Wuhan Aibotaike Company (Wuhan, China). Other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Animals

The study utilized forty SPF male sprague-dawley (SD) rats, aged 6–8 weeks and weighing approximately 200 ± 2 g. These rats were procured from the Experimental Animal Centre of Southern Medical University (Guangzhou, China), with a license number of SCK (E) 2020–0018 and a certificate number of 42000600046786. Prior to the experiment, the rats were housed under standard laboratory conditions at a temperature of 20°C-24 °C and a relative humidity of 45%–60 %, with a 12-h light/dark cycle. They were adaptively fed for 7 days before the commencement of the experiment. The animal experimental protocols were conducted in accordance with the guidelines of the Animal Care and Use Committee of Sichuan University.

The study adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and received approval

from the Research Ethics Committee of Sichuan University (Approval No. SCU231124001).

2.3. Establishment and grouping of the collagen-induced RA rat model

Bovine type II collagen (2 mg/mL, Chondrex Inc.) and complete Freund's adjuvant (5 mg/mL, Sigma) were mixed at a 1:1 ratio in equal volume to form an emulsion. The prepared mixture was injected 100 μl subcutaneously at a distance of 2 cm from the distal end of the tail root of rats and their left foot, and the second immunization was carried out subcutaneously 14 days later. The arthritis score of hind paws was recorded every 7 days after the second immunization with the scoring system of the previous study. On the 21st day of modeling, the successfully modeled rats with arthritis scores over 4 were randomly divided into 3 groups: the control group (FK), the DZGP group (FZ, 0.45 g/kg/d), and the methotrexate group (FM, 1.35 mg/kg).

2.4. Assessment of paw swelling and weight

The AI score is utilized to assess the success of the modeling. Seven days post-secondary immunization, the inflammatory state of the ankle and paw is evaluated based on a scoring standard of 0–4, where 0 indicates normal or no inflammation, 1 signifies slight redness and swelling, 2 represents severe swelling, 3 denotes redness and swelling below the ankle joint encompassing the entire paw, and 4 implies severe redness and swelling of both the knee joint and ankle joint, potentially with deformation. The left and right hind feet of the rats are scored separately, and if the combined score is 4 or more, the modeling is deemed successful. The weight and paw thickness of the rats are recorded every 3 days following the first immunization, and the living conditions of each group are regularly monitored [3].

2.5. Histopathological examination

The rats were sacrificed via decapitation, and the knee joints were removed of excess muscle tissue and then soaked in 4 % paraformaldehyde, dehydrated with 10 % EDTA, and embedded in paraffin. The wax block was cut into slices (5 μm) and stained with haematoxylineosin (HE) after paraffin embedding, all of which were observed with a microscope and recorded images using the Nikon TS2 optical microscope and high-resolution digital camera system (Nikon TS2, Tokyo, Japan) [5].

2.6. Sample collection of serum and knee joints of RA rats

All rats were fasted for 12 h before operation and only kept free drinking water. Rats in FZ were given oral gavage for the last time before collecting serum for 3 h. Weighed before operation, rats were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg, i. p.), and apical blood was taken by a biochemical negative pressure tube without additives. Followed by standing for 30 min, and centrifuging at room temperature of 3000 rpm for 10 min, collecting the supernatant, and storing it in a refrigerator at −80 °C until biochemical analysis. According to the manufacturer's protocols and instructions, the ELISA kits were used to determine the levels of TNF-α, IL-1β in rat serum.

2.7. Analysis of serum components by UPLC-MS/MS

We freeze-dry the collected serum samples with a freeze dryer and grind them into powder, and then use 70 % methanol extract to dissolve the powder to 100 g/1.2 mL. After fully dissolving, we put it in the refrigerator at −49 °C overnight. Subsequently, the sample was centrifuged for 10 min at 12,000 rpm, and then the supernatant was extracted, filtered with a microporous membrane (0.22 μm), and finally stored in a detection bottle to examine phenolic acids, alkaloids, lignans, coumarins, coolies, flavonoids, and ligands for UPLC-MS/MS analysis.

2.8. Qualitative analysis and data processing of metabolites

The quantification of metabolites was completed by multi-reaction detection mode analysis of triple quadrupole mass spectrometry. The MWDB database was used to qualitatively analyze the secondary mass spectrum information. Metabonomic data analysis was completed by Analyst 1.6.3. The SMILES structure information of blood components was obtained through the PubChem database, and SMILES were imported into Swiss Target prediction to predict the potential point where DZGP could act. A metabolic–target–component interaction network was then established through protein interaction (PPI) information. Finally, the network was visualized and analyzed using the STRING database and Cytoscape software. Differential metabolites were used as potential biomarkers in the OPLS-DA analysis.

2.9. Intestinal flora analysis

Immediately after blood sample collection, intestinal contents were collected, placed into a sterile tube, and frozen at −80 °C. The entire collection process was carried out in an ultra-clean workbench. The analysis of intestinal microorganisms was carried out on 16S rDNA sequencing and entrusted to Wuhan Maiteville Company. Bacterial DNA was extracted using an intestinal DNA extraction kit (Qiagen Fecal DNA Extraction Kit, Qiagen, Hilden, Germany) [8].

2.10. Statistical analysis

We mainly used the Kruskal-Wallis rank-sum test to detect species with significant abundance differences. The difference between the two groups was compared by one-way ANOVA. All statistical tests in this experiment were completed using SPSS (Version 26.0) software. and $p < 0.05$ and $p < 0.01$ were considered statistically significant.

3. Results

3.1. DZGP treatment alleviated arthritis symptoms of RA rats

To explore the treatment effects and side effect of DZGP, we use positive control and negative control. As shown in Fig. 1A, rats began treatment with DZGP and MTX on day 14 after primary immunization, and the effects on body weight, paw thickness, and arthritis score were evaluated once a week. The results show the growth rate of FX was significantly slower than that of FK, and the tow thickness and arthritis scores were higher in FX. HE staining clearly showed the synovial tissue of FX proliferated obviously, arranged disorderly, and there were a large numbers of inflammatory cells infiltration. DZGP treatment reversed these changes (Fig. 1B). Importantly, DZGP treatment significantly reduced the tow thickness and arthritis score, and the rats gained normal weight (Fig. 1C). These results show that treatment with DZGP can significantly reduce joint destruction in RA rats. It's worth noting that MTX can also relieve toe swelling, but it can't restore the weight. This suggested that DZGP had better clinical safety than MTX.

3.2. DZGP treatment decreased the levels of IL-1β and TNF-α in RA rats

The inflammatory pathways, such as TNF-α and IL-1β, were responsible for the development and progression of RA inflammation. The ELISA assay showed the serum concentrations of TNF-α and IL-1β in FX were higher than those in FK. After the intervention of DZGP and MTX, the cytokine level decreased significantly (Fig. 2), indicating DZGP could control the occurrence of inflammation in vivo like MTX.
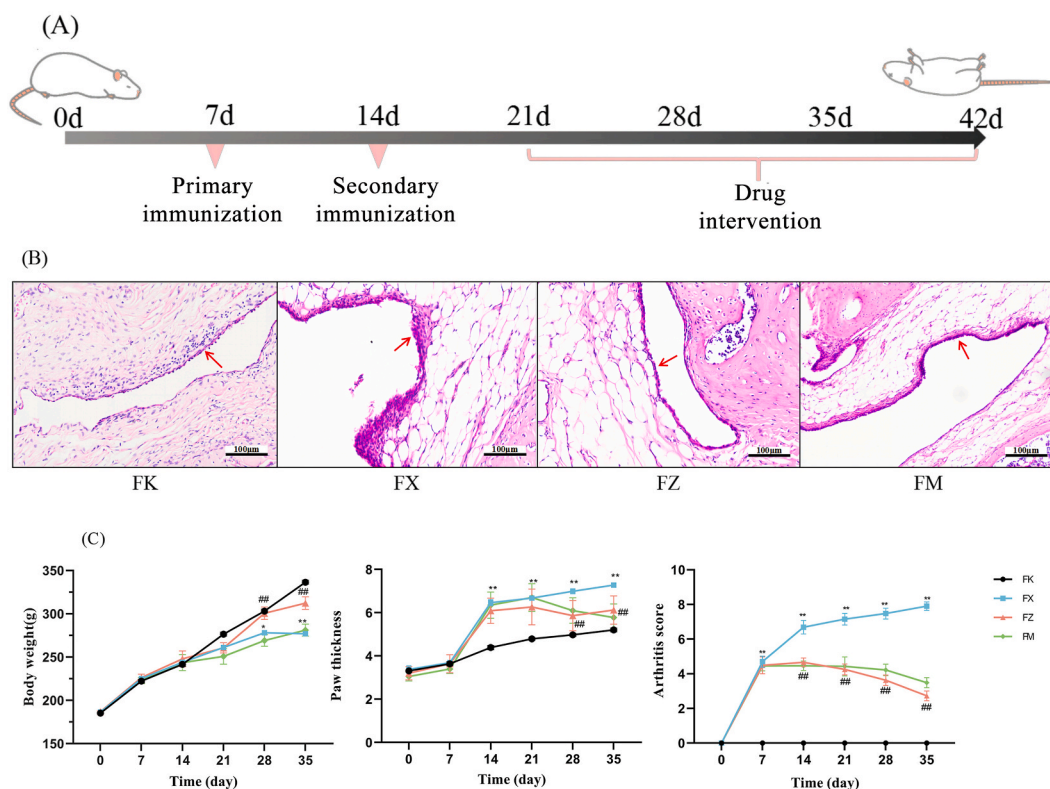
Fig. 1. DZGP inhibitd the histopathological changes in RA rats. and alleviated the severity of arthritis. (A) Experimental flow chart of this study. (B) eHE staining of synovial tissue 100× magnification. DZGP impact on changes in body weight, paw thickness and arthritis sore in RA rats. Data are presented as the means ± SD. n = 8.*$p < 0.05$ and **$p < 0.01$ (vs. FK), #$p < 0.05$ and ##$p < 0.01$ (vs. FX). (Note: FK:control group,FM:MTX group, FZ:DZGP group, FX:model group); The same as below).

3.3. UPLC/MS-MS analysis and identifcation of potential metabolites in DZGP treatment

Serum samples were analyzed using a total ion current diagram and a multi-reflection monitoring multi-peak diagram of triple quadrupole mass spectrometry. The results, shown in F**ig. 3A–D**, identified a total of 105 metabolites (Supplementary Table 1). These primarily include phenolic acids, alkaloids, lignans, coumarins, coolies, flavonoids, ligands, and others, with alkaloids, flavonoids, and phenolic acids being the most prevalent (Fig. 3E). Principal component analysis (PCA) demonstrated significant differences between the FZ and FX groups (Fig. 3F), and OPLS-DA analysis also indicated an independent distribution between FZ and FX (Fig. 3G). Both analyses suggest that we can effectively screen different metabolites. Ultimately, a total of 18 differential compounds were screened (Supplementary Table 2).

3.4. Network pharmacological analysis of blood components of DZGP

We obtained 390 predicted targets of blood components through the Swiss Target Prediction database. The intersection of predicted targets and RA-related targets is shown in Fig. 4A. Based on these 141 common targets, a "disease targets-blood components-traditional Chinese medicine" network was constructed (Fig. 4B). In this network, 3.4-diylflavone, 7.4-dihydroflavanol, a-ethyl-aminocinnamic acid, dehydroabietic acid, and 3,4-0-diccafé quinic acid methyl vinegar are the top five components, which may be key components for treating RA. Fig. 4C shows the interaction of common targets, with the yellow nodes representing the 10 core targets (namely, VEGFA, epidermal growth factor receptor (EGFR), TP53 etc.). AKT serine/threonine kinase 1 (AKT1), VEGFA, tumor protein p53 (TP53), EGFR proteins are involved in oxidative stress, inflammation, vascular permeability, and immune regulation, which may be related to RA pathogenies [29]. In addition, we counted the top 20 core targets of RA and the top 10 core targets of blood components (Supplementary Table 3). Their common core targets are VEGFA, TP53, EGFR, and AKT1, which are the main targets of DZGP in treating RA. Furthermore, GO and KEGG enrichment analyses found that they are mainly concentrated in the PI3K-AKT and Mitogen-activated protein kinase (MAPK) pathways (Fig. 4D). Additionally, simulated molecular docking results, as shown in Supplementary Table 4, indicate a good combination between blood components and core targets.

We conducted an analysis of the intestinal microflora structure in rats using 16S rDNA gene sequencing, yielding a total of 3,070,493 original sequences. Post-processing, we obtained 2,639,591 effective 16S rDNA tags, which account for 87 % of the original sequence. To check the impact of DZGP intervention on intestinal microflora, we examined the alterations in α diversity and β diversity post-DZGP treatment. The α diversity analysis revealed a significant difference in Shannon and Simpson indexes (Fig. 5A and B) when compared to FK. However, no significant difference was observed in the α-diversity indices for the Chaol and ACE indexes (Fig. 5C and D) when compared to FK. The diversity and species distribution uniformity of intestinal microbial communities in FX and FZ significantly increased compared to FK.

Further, the β diversity analysis results obtained from PCoA and NMDS analysis (Fig. 5E and F) clearly distinguished the four groups. These results suggest that the gut microbiota structure, specifically in Shannon and Simpson indexes, changed in the RA rats. The Venn diagram, representing the species richness table, highlighted the significant changes in intestinal microflora caused by DZGP in rats (Fig. 5G). Additionally, based on species annotation results, we selected the top 10 species in each sample or group at various taxonomic levels (Phylum (Fig. 5H) and Genus (Fig. 5I)) to generate the column accumulation of species relative richness. These results indicate a change in the community structure among the four groups of RA rats.

3.5. Analysis abundance of the gut microbiota structure at the phylum and genus levels

The LDA scores and evolutionary branches of differential microorganisms showed that the dominant phylum of FX were un-identified bacteria, while the dominant phylum of FK were Proteobacteria_9_Ralstonia and Euryarchaeot (Fig. 6A). Compared to FK,
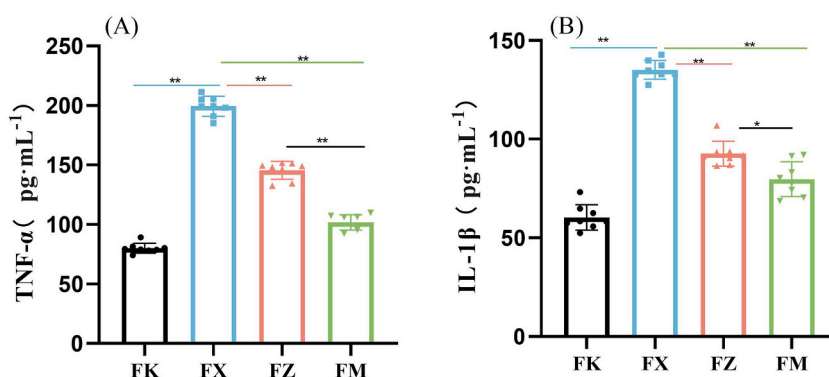

Fig. 2. DZGP treatment decreased serum inflammatory factor IL-1β and TNF-α levels. (A) Serum inflammatory factor TNF-α levels were detected using ELISA in different groups. (B) Serum inflammatory factor IL-1β levels were detected using ELISA in different groups. Data are presented as the mean ± SD. n = 8. *$p < 0.05$ means significant; **$p < 0.01$ means extremely significant.
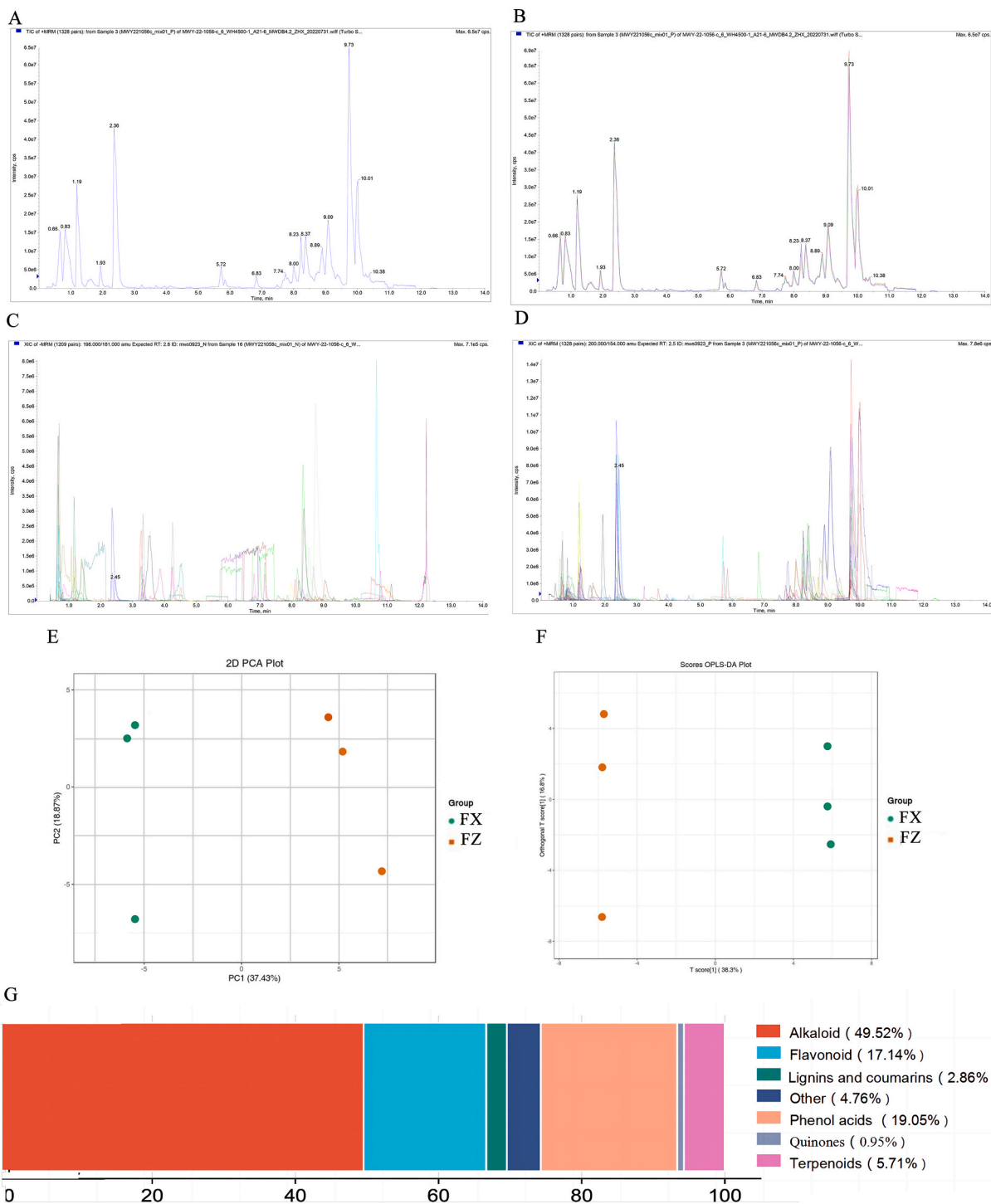
Fig. 3. Identification of serum components and screening of differential metabolites. (A)Negative ion mode of total ion flow pattern. (B) Positive ion mode of total ion flow pattern. (C) Negative ion mode of MRM. (D) Positive ion mode of MRM. (E) Group principal component analysis diagram. (F) OPLS-DA score graph. (G) Metabolite classes are composed of stacked plots.
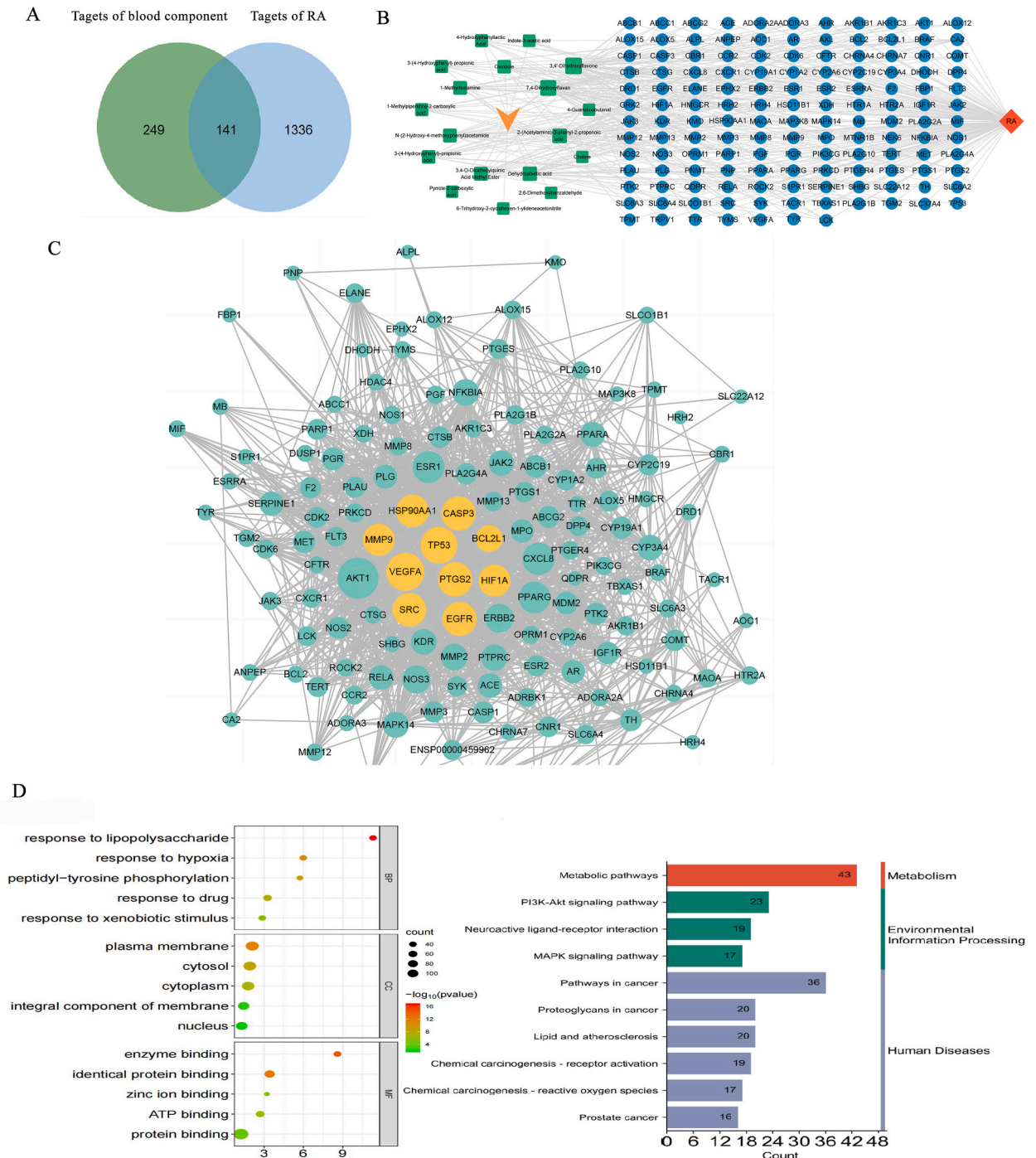
Fig. 4. The mechanism of DZGP inhibiting RA inflammation is related to suppressing PI3K-AKT and MAPK pathway. (A) Venn diagram of "component-disease" target intersection. (B) "Component-disease-target" Interaction network of DZGP. (C) PPI diagram for "component-disease" target screening. (D) GO enrichment and KGEE pathway annotations of potential treatment targets of DZGP.

the relative abundance of Eurarchaeota and Actinobacteria was relatively low in FX. Notably, the relative abundance of Bacterioidotais in FX increased significantly (vs. FK), but this trend was reversed in FZ (Fig. 6B). This suggests that DZGP primarily affects Eurarchaeota, Actinobacteria, and Bacterioidotais category bacteria.

Peptostreptococcaceae emerged as the dominant family in FX, while the control group was dominated by the genera Helicobacter, parasutterella, and bacteroides (Fig. 6C). When compared to FK, the relative abundance of parvibacter and Monoglobus was found to be lower in FX, but this trend was significantly reversed in FZ (vs FX) (Fig. 6D). In FX, following stimulation by the inducer, several
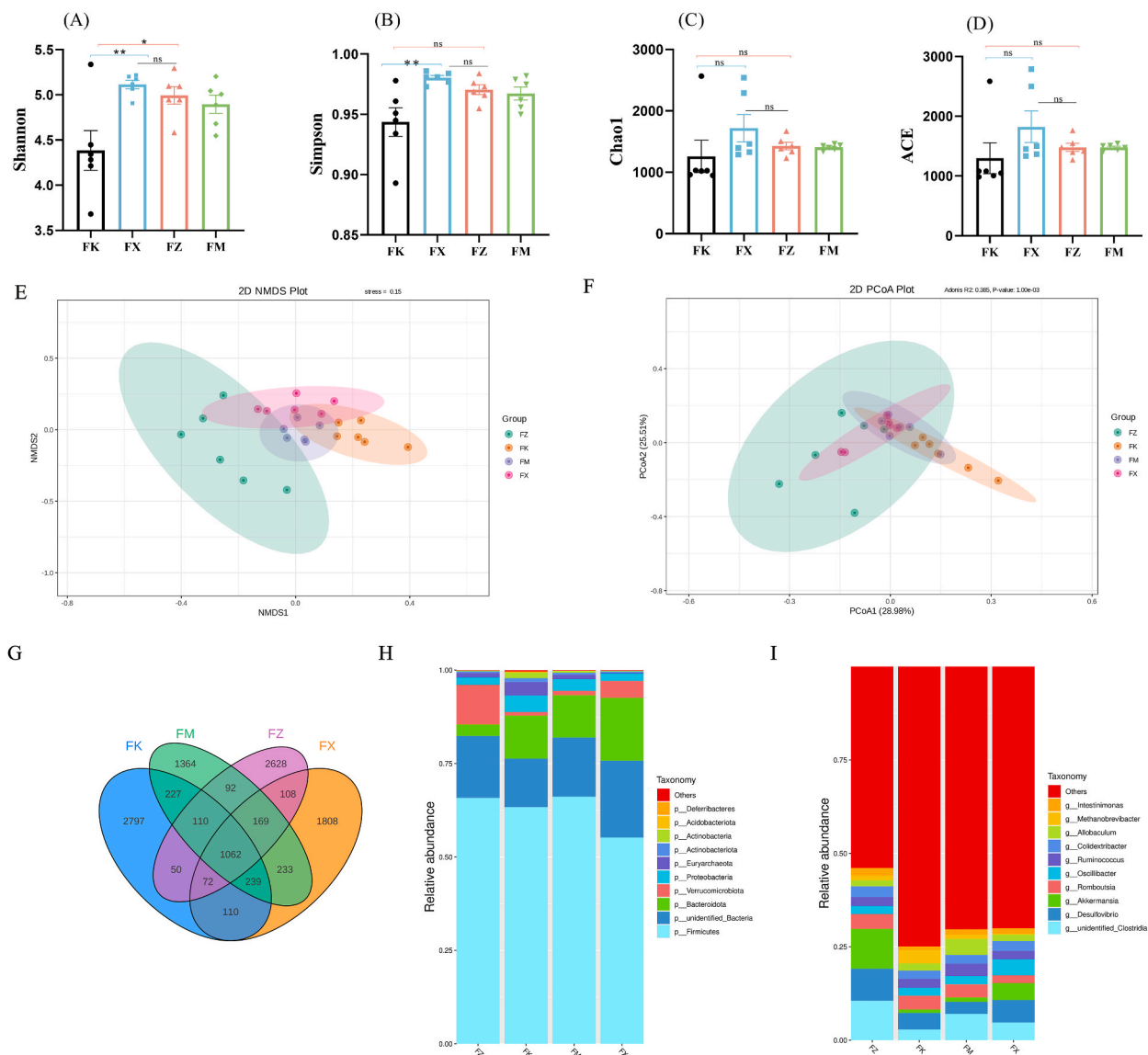
Fig. 5. DZGP regulates the structure of gut microbiota in RA rats. (A–D) Alpha diversity analysis by Shannon(A), Simpson(B), Chao 1 (C) and ACE index(D). (E) NMSP analysis of gut microbiota of rats among the four groups. (F)PCoA analysis of gut microbiota based on urneighted Unifrac. (G) Venn diagram among the four groups. (H)Column accumulation of species relative richness at Phylum level. (I)Column accumulation of species relative richness at Genus level. Data are presented as the mean ± SD. n = 6. *$p < 0.05$ means significant; **$p < 0.01$ means extremely significant; "ns" means not significant.

species in the intestinal tract of rats, including Bifidobacterium, Methanosphaera, Acetaficator, Alistipes, Anaeroplasma, Anaerotrun, Butyrichmonas, and Harriflinti, were induced. DZGP may alleviate intestinal inflammation and intestinal barrier injury induced by the inducer in FX, primarily by inhibiting Bacteroidota and restoring Monoglobus and Bifidobacterium.

Additionally, we predicted the influence of intestinal microflora function in rats. Based on these predictions, we selected the top 20 functional information items with the highest abundance in each KEGG classification level sample and created a bar graph of relative functional abundance (Fig. 6E). Across three levels of the KEGG database, the results indicated that DZGP primarily regulated cell metabolism through the following KEGG pathways: purine metabolism, carbon metabolism, biosynthesis of secondary metabolites, and biosynthesis of amino acids. The most highly enriched KEGG pathway in RA patients was amino acid metabolism [24]. These results suggest that the imbalance of some bacterial pedigree and the metabolic changes of intestinal microflora lead to a the change in the host immune model, which is key to RA pathogenesis.
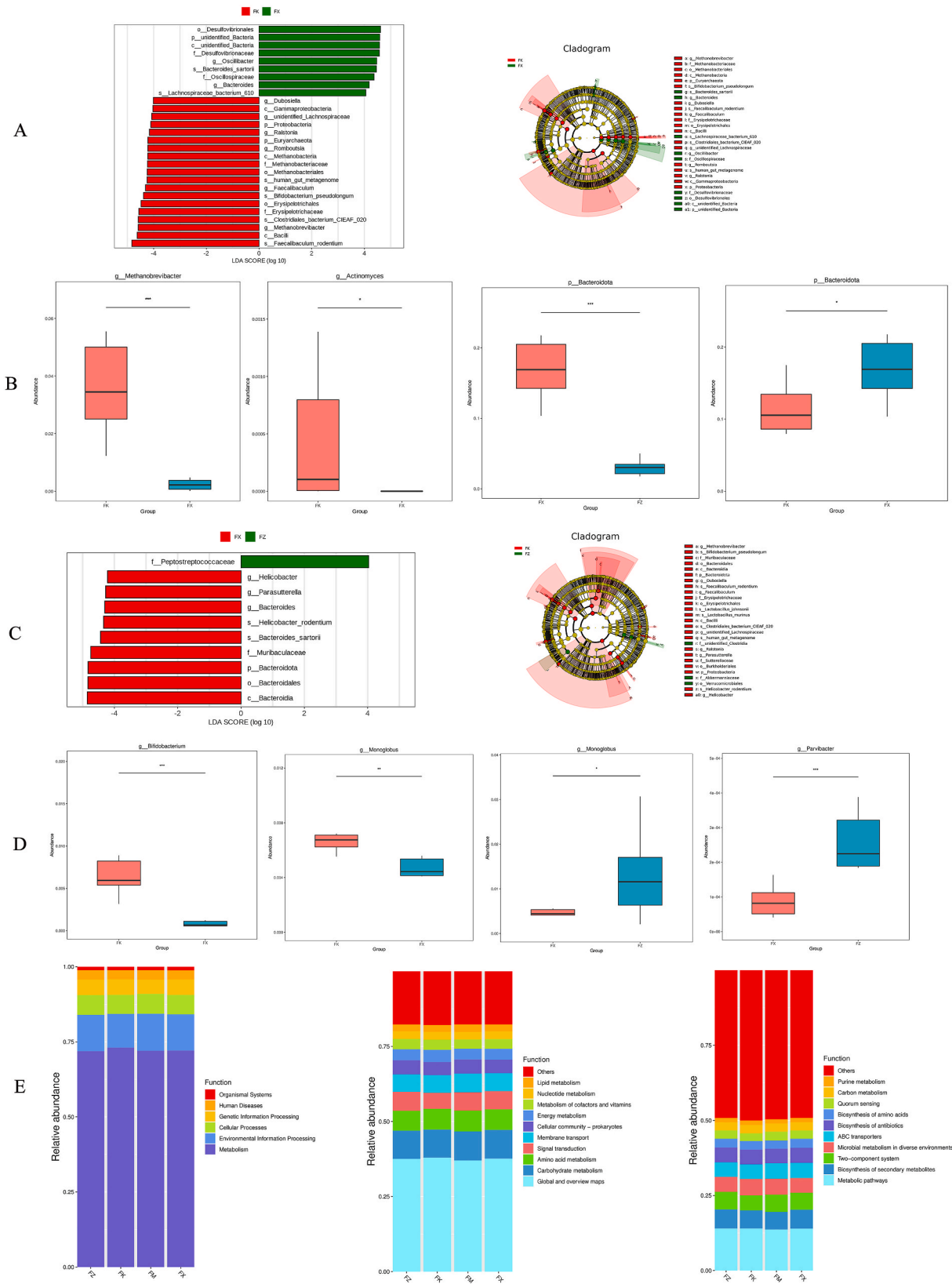
Fig. 6. Relative abundance of the gut microbiota structure at the phylum and genus levels. (A) LDA scores and evolutionary branches of differential microorganisms. (FX *vs* FK). (B) Statistical chart of Metastats significant differences between FK and FX at phylum levels. (C) LDA scores and evolutionary branches of differential microorganisms. (FX *vs* FZ). Statistical chart of Metastats significant differences between FX and FZ at genus levels. Column chart of relative abundance of each group of functional notes (Level 1–3). Data are presented as the mean ± SD. n = 6. *$p < 0.05$ means significant; **$p < 0.01$ means extremely significant; "ns" means not significant.

4. Discussion

As a chronic inflammatory and autoimmune disease, immune imbalance, inflammation, dysbiosis, and metabolic disorders are presented in RA pathologies, which promote irreversible joint damage and eventually lead to disability [32]. Most RA patients suffer from long-term administration of drugs such as MTX, GCs, and NASIDs, etc., which may cause some serious side effects and toxicities to the liver and kidney [33], Therefore, new, effective, cheap, and safe treatments are urgently required. DZGP exhibits various pharmacological effects, including diminishing pain and joint swelling and alleviating disease severity in experimental arthritis. In the Enshi area, DZGP has been used to treat RA for many years, and can effectively improve the patient's condition. However, the mechanism by which DZGP treats CIA is unknown. In our study, we identified the ingredients that can smoothly enter the blood DZGP, including the effective anti-inflammatory components of DZGP. To a certain extent, this breaks through the limitation of people's understanding of the effective components of DZGP in the past. It also provides a theoretical basis for the clinical application of DZGP for treating RA.

In this study, we used collagen-induced rats as a negative control and MTX-treated rats as a positive control, and dose was based on previous studies [34]. We observed that DZGP can significantly improve the quality of life of rats with RA, reduce the inflammatory reaction of joints, and reduce the expression of TNF-α and IL-1β. Joint inflammation is mediated by increased pro-inflammatory and decreased anti-inflammatory cytokines. The former, such as TNF-α, IL-6, and IL-1β, play a particular role in the pathogenesis of bone erosion [35]. In line with previous studies, DZGP treatment significantly decreased inflammatory cytokines, thus alleviating joint swelling.

Existing research indicates that DZGP can foster the growth and healing of bone tissue in various ways. For instance, it can enhance the expression of AKT, VEGF, BMP-2, BMP-7, BMP-9, and Smad 1/5 genes in bone tissue [36–39], while suppressing the expression of AKT and Smad-6 genes. Additionally, DZGP can promote bone tissue through methods such as reducing blood calcium and increasing blood phosphorus.

However, due to the complex composition of DZGP, current research on its effective components is limited, making it challenging to fully comprehend the mechanisms of DZGP. This also hinders the production quality control of DZGP.

We analyzed the secondary metabolites in the drug-containing serum of rats and identified 18 different metabolites. 3.4-di-light-flavone, 7.4-dihydroflavanol, a-ethylcool aminocinnamic acid, dehydroabietic acid, and 3.4-0-dicafeku quinic acid Jiaku played the most significant role in the blood components of DZGP. GO analysis revealed that the target of DZGP in the treatment of RA could regulate the body's response to lipopolysaccharide and hypoxia, increase the activity of MAPK kinase, and affect the phosphorylation of skin tyrosine. These effects are related to cell proliferation, vasoconstriction, and apoptosis. Our research results provide some scientific basis for the clinical application of DZGP and offer directions and ideas for future research.

Abnormal proliferation of synovial fibroblasts is a primary pathological change in RA [40]. This abnormality, coupled with the abnormal activation of the PI3K-Akt signaling pathway, inhibits the apoptosis of synovial fibroblasts [41,42]. AKT1, a key node in the PI3K-Akt signaling pathway, regulates numerous processes, including metabolism, proliferation, cell survival, growth, and angiogenesis [43,44]. Its abnormal activation reduces the dimer formed by Bad and Bcl-xl, thereby inhibiting cell apoptosis and leading to synovial fibroblasts. VEGFA, a member of the platelet-derived growth factor family, promotes the proliferation of vascular endothelial cells when activated. While the expression level of VEGF is low in normal tissues, it is often overexpressed in the synovial tissues of RA patients. VEGF can also inhibit the normal apoptosis of cells by inducing the expression of the Bc1–2 protein [45–47]. EGFR, the first subtype of the tyrosine kinase receptor family, can activate the PI3K/AKT signaling pathway and up-regulate VEGF upon binding to related ligands, thus causing abnormal apoptosis [48]. Recent research shows that DZGP can inhibit osteoclast differentiation and prevent RA-induced bone destruction by down-regulating AKT. However, further research is needed on how DZGP affects the expression of VEGFA and EGFR in RA patients.

MAPK is an important signal system that mediates cell responses. MAPK is closely related to cell proliferation, differentiation, apoptosis, and cell function. The activation of MAPK is closely related to the inflammatory reaction in RA. Among them, JNK/SAPK and p38 are signal pathways related to inflammation. The inflammatory reaction can be reduced by using the p38 inhibitor SB203580 and the JNK inhibitor SP600125 [49,50]. Additionally, MAPK is upstream of the NF-κB signaling pathway, and NF-κB is widely involved in specific inflammatory processes by activating immune cells [51]. Therefore, regulating the MAPK signaling pathway can avoid the activation and expression of the NF-κB signaling pathway. The subsequent intermediate signal molecules E2F2 and EP1-4 will form a swelling medium for joint pain, aggravate the infiltration of inflammatory cells, and promote the proliferation and differentiation of osteoblasts [52]. In addition, FLS proliferate, enhance their invasion and infiltration abilities, and reduce apoptosis, while activated NF-κB promotes FLS proliferation [53]. In this study, DZGP significantly decreased TNF-α and IL-1β in the serum of RA rats, suggesting that MAPK may be one of the mechanisms.

TP53 plays a crucial role in regulating cell life activities, including cell proliferation, apoptosis, and angiogenesis [54]. Studies have shown that certain p53 abnormalities identified in the pathogenic cells of RA are associated with their abnormal behavior, such as resistance to apoptosis, abnormal survival, and invasiveness [55,56]. Given that apoptosis is a fundamental regulator of innate and

adaptive immune responses, damaged apoptosis of pathological cells appears to play a significant role in the abnormal cell survival that leads to disease development [57]. The loss of mutant p53 function may be associated with the detrimental effects of cytokines and metalloproteinases produced in the rheumatoid synovium. Ultimately, mutations in the TP53 gene contribute to the maintenance of inflammation and its progression to chronic disease, serving as a marker of RA [58,59]. A small amount of TP53 protein and TP53 mRNA have been detected in PBMCs from RA patients [60]. Additionally, p53 downstream target genes such as p21, growth arrest, and DNA damage-induced alpha, and p53 apoptosis effectors related to PMP-22 were down-regulated in RA PBMCs [61].

TP53 deficiency in RA-FLSs plays a significant role [62]. The dysfunction of p53 in FLSs can induce resistance to apoptosis by overexpressing XIAP and reducing pro-apoptotic p53 target genes such as PUMA and P53AIP1 [63]. The addition of MIF in RA-FLSs enhances HIF-1α by inhibiting p53 function. The increase in HIF-1α induces VEGF production and angiogenesis [64]. Furthermore, defective p53 in RA-FLSs can induce chronic inflammation in the synovium. Damaged p53 in RA-FLSs leads to the upregulation of MAPK-activated protein kinases, including MAPK, which results in the activation of the NF-κB and MAPK pathways and an enhanced inflammatory response [65]. Defective p53 leads to reduced expression of p21 and increased inflammation through excessive production of IL-6 and MMPs [66]. Additionally, damaged p53 increases the expression of Cyr61, which plays a role in increased inflammation through the activation of the Integrin and Akt/NF-κB pathways [67].

The pathogenesis of RA has traditionally been attributed to immune, genetic, and environmental factors [68]. Recent studies have confirmed the involvement of the microbiota in the pathogenesis of RA [69]. Studies have shown an altered composition of the microbiota in early RA patients, demonstrated that Prevotella species were dominant in the intestines of patients in the preclinical stages of RA. In addition, Prevotella-dominated microbiota isolated from RA patients contributes to the development of Th17 cell-dependent arthritis in SKG mice [70]. With advancements in science and technology, we can now determine the composition of the intestinal flora in each group of rats using 16S rDNA sequencing technology. The results of PCA and PCoA analyses reveal a clear separation between the model group and the control group, indicating a change in the intestinal microbial composition of RA rats. This conclusion aligns with a study on human RA, which shows significant differences in intestinal microflora between RA patients and healthy control groups [71].

Our correlation analysis suggests that DZGP modulates the gut microbiota, which may play a role in regulating PI3K-Akt and MAPK signaling pathways and improving RA. Recent reports have shown that the PI3K-Akt and NF-κB/MAPK signaling pathways can be regulated at several levels, including the gut microbiota. For instance, enterotoxins produced by bacteria can suppress apoptosis by regulating the MAPK pathway [72]. Some metabolites, such as butyrate and propionate produced by Firmicutes and Bacteroidetes, are correlated with apoptosis. Butyrate promotes cell growth through proliferation and apoptosis inhibition, while propionate has been reported to induce apoptosis by increasing the expression of mitochondrial apoptotic pathway-related proteins [73].

However, our study has some limitations. Factors such as diet, housing conditions, and handling procedures could affect the results. The pathway through which DZGP alters gut microbiota to alleviate RA remains to be explored. Furthermore, the application of DZGP in RA patients and the safety and efficacy of this approach are yet to be determined. Lastly, apart from methotrexate (MTX), there are emerging biological agents, and the advantages of DZGP compared to these new biological agents need to be elaborated.

5. Conclusion

Our research revealed that DZGP could relieve RA rats' symptoms and showed lower toxicity. At the same time, it showed a good anti-inflammatory effect on RA and played an important role in improving the structure of the gut microbiota, which enriched DZGP's fundamental research on the immune, metabolic, and microorganism aspects of RA. Taken together, our data provide compelling proof for a previously undescribed role of DZGP in RA treatment and lay a solid foundation for DZGP as a potential treatment for RA.

Data availability statement

The authors do not have permission to share data.

Ethics declarations

The study was in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Research Ethics Committee of Sichuan University (NO·SCU231124001).

CRediT authorship contribution statement

Liming Zhao: Writing – original draft, Project administration, Conceptualization. **Meilin Liu:** Writing – review & editing, Methodology, Data curation. **Kai Zheng:** Formal analysis, Data curation. **Qiang Xiao:** Methodology, Formal analysis. **Lin Yuan:** Software, Formal analysis. **Chuanfang Wu:** Supervision, Project administration, Funding acquisition. **Jinku Bao:** Visualization, Resources, Project administration, Funding acquisition.

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.

Acknowledgments

This work was funded by The Open Fund of Hubei Key Laboratory of Biological Resources Protection and Utilization (Hubei Minzu University) (KYPT012302); and was supported by the National Natural Science Foundation of China (Grant No. 31971162; Grant No. U20A20410; Grant No. 32071275).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32705.

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