Am J Transl Res 2024;16(2):654-668
www.ajtr.org /ISSN:1943-8141/AJTR0150978

Original Article

Synergistic impact of Gui Zhi Shao Yao Zhi Mu Decoction and leflunomide on gut microbiota in rheumatoid arthritis: insights from 16S rDNA sequencing

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Received April 29, 2023; Accepted December 27, 2023; Epub February 15, 2024; Published February 28, 2024

Abstract: Background: Rheumatoid arthritis (RA) is a chronic autoimmune disease with complex pathogenesis, including alterations in the gut microbiota. Gui Zhi Shao Yao Zhi Mu Decoction (GSZD), a traditional Chinese herbal formula, has shown efficacy in RA treatment, but its impact on intestinal microflora remains unclear. This study aimed to investigate the effects of GSZD combined with leflunomide on the gut microbiota of RA patients. Methods: The study enrolled 48 RA patients who were randomly assigned to either a control group receiving leflunomide or a treatment group receiving GSZD combined with leflunomide for 12 weeks. Gut microbiota composition was analyzed pre- and post-intervention using 16S rDNA sequencing. Changes in microbial diversity, abundance, and metabolic functions were assessed. Results: Post-treatment, both groups exhibited significant alterations in gut microbiota composition. GSZD combined with leflunomide led to an increased Bacteroidetes/Firmicutes ratio and a reduction in Actinobacteria compared to leflunomide alone. This was associated with beneficial shifts in microbial genera and metabolic pathways, suggesting improved gut health and systemic immune modulation. Conclusion: GSZD combined with leflunomide significantly modulates the gut microbiota in RA patients. This study provides insights into the mechanisms underlying the therapeutic effects of GSZD and highlights the potential of integrating traditional Chinese medicine with conventional treatments in managing RA.

Keywords: Gui Zhi Shao Yao Zhi Mu Decoction, rheumatoid arthritis, intestinal microflora, 16S rDNA sequencing, leflunomide

Introduction

Rheumatoid arthritis (RA) is a debilitating chronic autoimmune disorder with a global prevalence of about 0.32-1%, representing a significant public health challenge [1-3]. Characterized by progressive joint inflammation, RA leads to substantial morbidity, functional impairment, and decreased quality of life. Additionally, it increases the vulnerability of several diseases, particularly colorectal tumors [4-6].

The disease's etiology is multifactorial, involving a complex interplay of genetic predisposition, environmental triggers [7, 8], and immunological dysregulation [9]. Genetic susceptibility plays a crucial role in RA pathogenesis. Research over the past few decades has identified multiple genetic loci associated with an increased risk of developing RA. The HLA-DRB1 shared epitope alleles are among the most significant genetic factors, with studies demonstrating their strong association with disease susceptibility and severity [10]. Other notable genes include PTPN22, which encodes a tyrosine phosphatase involved in T cell receptor signaling, and STAT4, a transcription factor critical for signaling in various immune cells and inflammatory diseases [10, 11]. These genes contribute to the intricate network governing immune responses, influencing both the initiation and progression of RA.

https://doi.org/10.62347/TDTJ1944

In addition to genetic factors, the role of the gut microbiome in RA has garnered significant attention. The human gut harbors a complex community of microorganisms, which play a vital role in health and disease. Imbalances in this microbial ecosystem, known as dysbiosis, have been implicated in various autoimmune conditions, including RA [12]. Patients with RA commonly exhibit an altered gut microbiota composition, characterized by decreased microbial diversity and a shift in specific bacterial populations. Notably, there is often a decline in anti-inflammatory bacteria such as Faecalibacterium and an increase in pro-inflammatory genera like Prevotella [13]. This dysbiosis is believed to contribute to the pathogenesis of RA by promoting systemic inflammation and autoimmunity.

Emerging evidence suggests that pharmacological interventions can influence the gut microbiota composition in RA patients [14-16]. Disease-modifying antirheumatic drugs (DMARDs), including methotrexate and leflunomide, have been shown to modify gut microbiota, potentially exerting therapeutic effects beyond their known immunomodulatory actions [17, 18]. These drugs seem to restore a more balanced microbial environment, enhancing the abundance of beneficial bacteria and suppressing harmful strains, thereby potentially reducing inflammation and alleviating RA symptoms [19-22].

The integration of traditional Chinese medicine (TCM) in RA treatment offers an interesting dimension. TCM, with its holistic approach and emphasis on restoring balance, has been used for centuries to manage chronic diseases [23-25]. Gui Zhi Shao Yao Zhi Mu Decoction (GSZD) is a classic TCM formula that has demonstrated efficacy in treating RA. It encompasses a range of pharmacologically active components that synergistically target multiple pathological pathways in RA [26]. Notably, TCM formulations like GSZD are generally associated with low toxicity and minimal side effects, making them an attractive option for long-term management of chronic conditions like RA [27].

The innovative aspect of this study lies in exploring the combination of GSZD with leflunomide, a standard DMARD, to treat RA. This combination therapy represents a convergence of traditional wisdom and modern pharmacology. Such integrative approaches are gaining traction in the realm of RA treatment, offering the potential to enhance therapeutic efficacy, mitigate adverse effects, and improve patient outcomes [28]. The rationale behind combining GSZD with leflunomide stems from their complementary mechanisms of action. While GSZD addresses the underlying imbalances and systemic inflammation characterizing RA, leflunomide directly targets the immune system's aberrant responses. This dual approach could offer a more comprehensive management strategy for RA.

Our study aims to investigate the effects of this combination therapy on the gut microbiota of RA patients, using 16S rDNA sequencing technology. We hypothesize that the synergistic action of GSZD and leflunomide not only modulates the immune response but also positively influences the gut microbiome, thereby offering a more holistic approach to RA management. This research could pave the way for novel treatment paradigms in RA, emphasizing the importance of considering both the microbiome and immune system in developing effective therapeutic strategies.

Materials and methods

Study population

Between January 2020 and December 2022, we recruited 48 participants from the rheumatology clinic at the Shunyi Hospital of Beijing Hospital of Traditional Chinese Medicine. These participants, aged 18-65, met the 1987 American College of Rheumatology and 2010 European League Against Rheumatism diagnostic criteria for rheumatoid arthritis. Eligible individuals had arthritis without organ damage or comorbidities and had not used medication for at least one week. This group included both first-time and non-continuously medicated patients. Informed consent was obtained from all participants, with 42 completing the study. The study was approved by the local ethics committee.

Intervention

Prior to treatment, we collected baseline characteristic indicators and stool samples from the 42 participants. We then randomly divided them into two groups using a random grouping

table and sealed, coded envelopes. Patients with odd numbers were allocated to the control group, receiving oral Leflunomide (State Drug quantification H20000550 lot 210503), while those with even numbers formed the experimental group, receiving both oral Leflunomide and GSZD. The GSZD formula, prepared by the pharmacy at Shunyi Hospital, included Gui Zhi 12 g, Red Peony 9 g, Licorice 6 g, and Ephedra 6 g, et al. The herbs were decocted in a specific sequence and duration, then packaged for use. After 12 weeks of treatment, we reassessed the clinical characteristics and collected stool samples again.

Sample collection

Participants collected mid-stream stool samples between 7:00 and 9:00 a.m. in sterile containers. Using sterile swabs, samples were taken from the center of the stool and placed in sterile lyophilization tubes, then immediately frozen in liquid nitrogen and stored at -80°C.

Microbiome analysis based on 16S rDNA gene sequencing

DNA was extracted from fecal samples using a Qiamp Fast DNA Stool extraction kit from Qiagen. The DNA's quality and concentration were verified using agarose gel electrophoresis and Nanodrop techniques. PCR amplification utilized Pfu high-fidelity DNA polymerase from All-Style Gold, and magnetic beads from Vazyme for purification. The amplification products were quantified using a Microplate reader and a Quant-iT PicoGreen dsDNA Assay Kit. Library preparation employed Illumina's TruSeq Nano DNA LT Library Prep Kit, with quality assessed on an Agilent Bioanalyzer using the Agilent High Sensitivity DNA Kit. Libraries were quantified using the Promega QuantiFluor fluorescence quantification system, with a concentration of 2 nM or higher for sequencing.

Data analysis and statistics

In our study, statistical analyses were conducted using SPSS version 21.0. For data following a normal distribution, independent and paired t-tests were applied. The independent t-test compared mean values between two independent groups, such as the LEF and LEGZ groups, while the paired t-test was used to compare mean values within the same group before and after treatment. For data not normally distributed, non-parametric rank sum tests (Mann-Whitney U test for two independent samples and Wilcoxon signed-rank test for paired samples) were employed. The Chi-square (χ^2) test was used for analyzing count data, such as the frequency of occurrence of specific taxa in the gut microbiota, to determine if there were significant differences in the distribution of categorical variables between groups. Statistical significance levels were set at *p*-values of 0.05, 0.01, and 0.001, with a *p*-value less than 0.05 considered statistically significant. For multiple comparisons, Bonferroni correction was applied to adjust the *p*-values, thereby reducing the likelihood of Type I errors (false positives). Participants who missed mid-trial visits, affecting sample collection, were excluded from the analysis to maintain data integrity. The incidence of adverse reactions was calculated as a percentage of total cases, providing a straightforward measure of the frequency of adverse effects associated with the treatments.

Results

General characteristics of the study cohort

This study initially included 48 patients, with successful completion of clinical observations in 42 cases. Withdrawals occurred due to two reasons: intolerability to the taste of Chinese medicine, leading to three withdrawals, and liver function impairment, accounting for another three withdrawals. Consequently, the incidence of adverse reactions was noted at 12.5%. The LEF (Leflunomide) control group comprised 21 patients, as did the LEGZ (Leflunomide combined with Gui Zhi Shao Yao Zhi Mu Decoction) trial group. For clarity, the pre-treatment phase in the LEF group was labeled as RA1, and in the LEGZ group as RA2.

The focus of the study was a comparative analysis of clinical characteristics between the two groups, both pre- and post-treatment. Detailed in **Table 1**, we observed a reduction in inflammatory markers such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels, and rheumatoid factor (RF) in both groups following treatment. Notably, the LEGZ group exhibited a more pronounced decrease in these indices. Particularly, the significant reduction in the RF index in the LEGZ group indicates a

Table 1. Clinical characteristics of patients in both groups before and after treatment

Clinical factor	RA1; n=21	RA2; n=21	LEF; n=21	LEGZ; n=21
ESR, mm/h	33.95±3.38	50.57±5.74**	30.81±4.34	30.23±3.91ΔΔ
CRP, mmol/L	7.95±1.38	18.57±2.86**	6.90±1.92	5.72±1.75Δ
RF, IU/mL	300.86±95.34	227.21±58.28	326.86±114.93	114.79±35.68
Pressure pain	4.33±0.25	4.24±0.30	2.24±0.25**	0.57±0.21ΔΔ,▲▲
Swelling score	3.29±0.40	3.43±0.35	1.86±0.31*	0.48±0.19ΔΔ,▲▲
Rest pain score	3.67±0.34	4.24±0.34	2.29±0.29**	1.14±0.20ΔΔ,▲▲
Ritchie	5.81±0.72	4.24±0.34	2.29±0.29*	1.14±0.20ΔΔ,▲▲
American rheumatoid arthritis index	5.76±0.83	8.67±1.53	3.95±0.57	2.14±0.58ΔΔ,▲▲
DAS28 score	3.04±0.04	3.07±0.06	2.63±0.15**	1.74±0.12ΔΔ,▲▲
CDAI	16.67±0.96	16.95±1.32	11.86±0.92**	6.10±0.96ΔΔ,▲▲
SDAI	17.46±0.97	18.81±1.53	12.55±0.96**	6.67±0.98ΔΔ
HAQ	7.10±0.50	10.90±0.84	4.86±0.61*	3.24±0.64ΔΔ,▲

Note: Statistically significant differences compared to the RA1 group *P < 0.05, **P < 0.01. Statistically significant differences ΔP < 0.05, ΔΔP < 0.01 compared with the RA2 group. Compared with the LEF group, the differences were statistically significant ▲P < 0.05, ▲▲P < 0.01. DAS28: Disease Activity Score 28. RA1: pretreatment group for the LEF (Leflunomide) control group, RA2: pretreatment group for the LEGZ (Gui Zhi Shao Yao Zhi Mu Decoction combined with Leflunomide) experimental group.

potential for improved prognosis in patients with high-titer RF.

Before treatment, there were no significant differences between the groups in various clinical indices, including the visual analog scale (VAS) for pressure pain, swelling score, rest pain score, Disease Activity Score 28 (DAS28), Ritchie Articular Index, American Rheumatoid Arthritis Index, Clinical Disease Activity Index (CDAI), Simplified Disease Activity Index (SDAI), and Health Assessment Questionnaire (HAQ) ($P > 0.05$). Post-treatment, however, both groups showed a decline in these indices, with the LEGZ group demonstrating significantly greater improvement than the LEF group. These findings indicate that the addition of GSZD to Leflunomide in the LEGZ group was more effective in enhancing clinical outcomes compared to the LEF group, suggesting the superiority of the combination therapy over Leflunomide monotherapy in the management of rheumatoid arthritis.

Description of sequencing results

In this comprehensive study, we employed 16S rRNA gene sequencing to meticulously analyze stool samples, aiming to explore the potential influences of Gui Zhi Shao Yao Zhi Mu Decoction on the modulation of gut microbiota. Notably, our sequencing endeavors revealed a substantial initial data volume of 118,789.8. Following the meticulous removal of chimeric sequences, the resulting dataset comprised 80,976.76 sequences, each with an average length of approximately 405 base pairs from Table S1. This robust dataset enabled a thorough evaluation of the microbial landscape within our samples.

Our analysis was further enriched by the application of diversity indices, a critical tool in understanding ecological complexity. The diversity index coefficient curve, as depicted in **Figure 1A**, presents a compelling narrative: as the number of sequences reached the threshold of 20,000, the curve plateaued. This observation suggests that beyond this point, additional sequencing would yield diminishing returns in terms of discovering new microbial taxa, thus implying that our sequencing depth was adequate for capturing the majority of microbial diversity within each sample group.

Complementing this, our analysis of the Shannon diversity curve, also illustrated in **Figure 1A**, echoed these findings. The plateau observed in this curve further reinforces the conclusion that our sequencing effort was sufficiently comprehensive to encompass the vast majority of the microbial diversity present in the samples.

An aspect of paramount importance in our study was the Operational Taxonomic Unit (OTU) coverage, which exceeded 98% across all groups, as detailed in Table S2. This high level

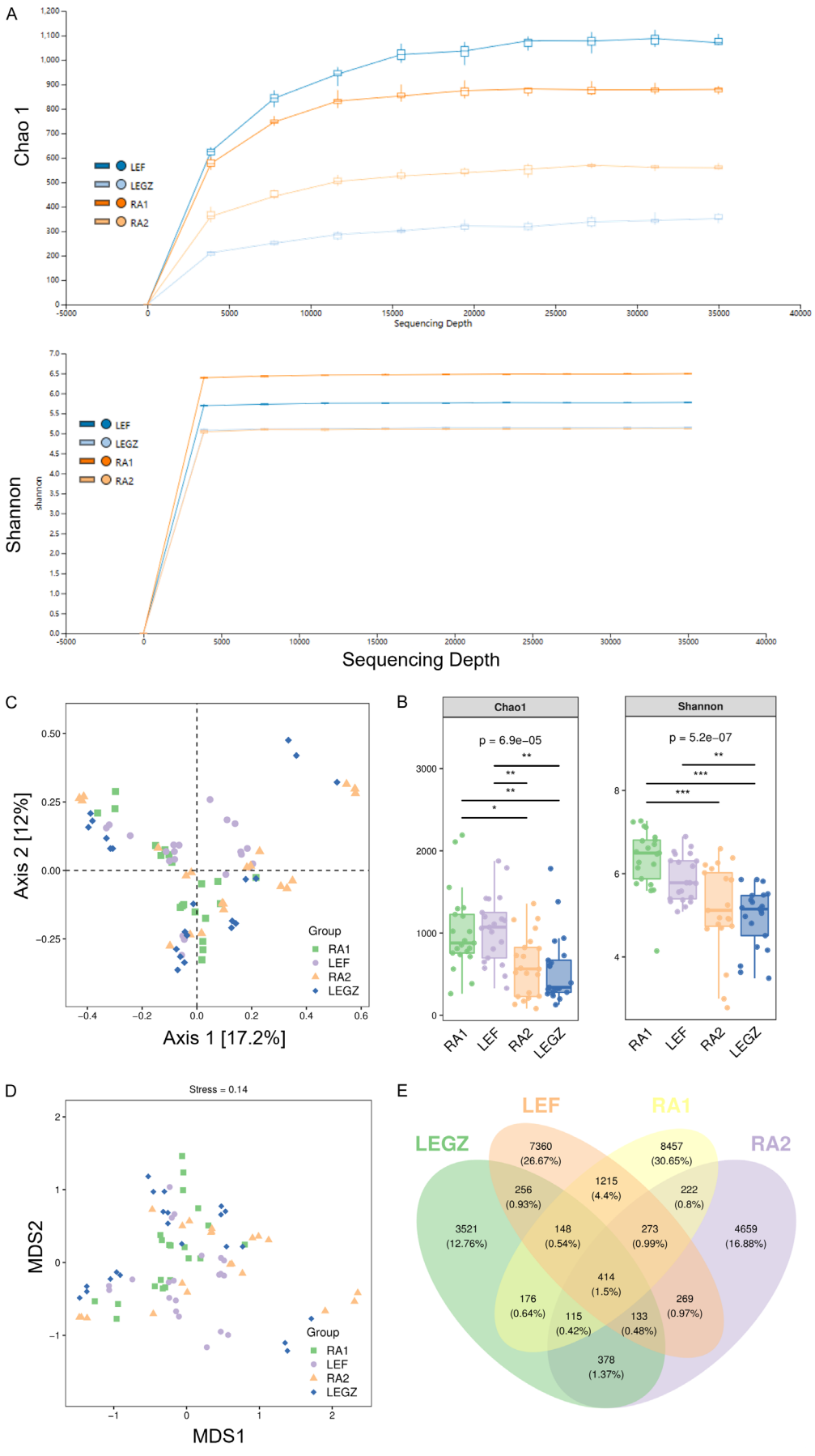

A
Chao 1
LEF
LEGZ
RA1
RA2
Sequencing Depth
Shannon
Sequencing Depth
C
Axis 2 [12%]
Axis 1 [17.2%]
Group
RA1
LEF
RA2
LEGZ
B
Chao1
p = 6.9e−05
Shannon
p = 5.2e−07
RA1
LEF
RA2
LEGZ
D
Stress = 0.14
MDS2
MDS1
Group
RA1
LEF
RA2
LEGZ
E
LEF
RA1
LEGZ
RA2
7360 (26.67%)
8457 (30.65%)
1215 (4.4%)
256 (0.93%)
222 (0.8%)
3521 (12.76%)
148 (0.54%)
273 (0.99%)
4659 (16.88%)
414 (1.5%)
176 (0.64%)
115 (0.42%)
133 (0.48%)
269 (0.97%)
378 (1.37%)

Figure 1. A. Dilution curves of intestinal flora, including Chao1 curve and Shannon curve. B. Alpha diversity of intestinal flora composition: Chao1 index and Shannon index. C, D. β-diversity analysis of intestinal flora composition: unconstrained PCoA and NMDA based on weighted Unifrac index (pressure =0.14). E. Venn diagram of OUT between groups. *P < 0.05, **P < 0.01 and ***P < 0.001. RA1: pretreatment group for the LEF (Leflunomide) control group, RA2: pretreatment group for the LEGZ (Gui Zhi Shao Yao Zhi Mu Decoction combined with Leflunomide) experimental group.

of coverage is indicative of an exceptionally high microbial detection rate within our samples. Such extensive coverage is crucial, as it ensures that our analyses are representative of the true microbial diversity present in the samples. This level of coverage not only meets but exceeds the rigorous standards required for both sequencing fidelity and subsequent database analysis, bolstering the reliability of our findings.

Diversity analysis of intestinal flora

In our exploratory research, we conducted a comprehensive diversity analysis, encompassing both α-diversity and β-diversity, to evaluate the impact of GSZD combined with Leflunomide on the intestinal flora of patients with Rheumatoid Arthritis (RA). α-Diversity is typically indicative of the richness and variability within the intestinal microbiome. Our findings, as depicted in **Figure 1B**, utilized the Chao1 and Shannon indices for assessment. These indices revealed that there were no statistically significant alterations in the abundance or diversity of the intestinal microorganisms between the LEF and LEGZ groups, either before or after the treatment ($P > 0.5$). β-Diversity, which serves as a measure of the community's similarity and diversity across different groups, was also evaluated. The analysis utilized principal coordinates analysis (PCoA) and a NMDS based on an unweighted distance matrix, as shown in **Figure 1C** and **1D**. These analyses further corroborated the absence of significant differences in the overall composition of the intestinal microbiota among the studied groups. However, an intriguing aspect was uncovered in the Venn diagram analysis (**Figure 1E**), illustrating that while most operational taxonomic units (OTUs) were shared across all groups, unique OTUs were identified in the LEF and LEGZ groups, numbering 7360 and 3521, respectively. This observation suggests subtle but potentially meaningful shifts in the microbial communities, attributable to the treatments. These unique OTUs might play a role in the gut microbiome's response to RA and its treatment, warranting further investigation.

Composition and alteration of the intestinal flora

In our pursuit to understand the intricate bacterial ecosystems within rheumatoid arthritis (RA) patients, we conducted a detailed analysis of the relative abundance of bacterial taxa at both the phylum and genus levels, examining their community structures. Our findings revealed that the gut microbiota in each examined group predominantly comprised phyla such as Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, among others (**Figure 2A**). Notably, Firmicutes and Bacteroidetes emerged as the most abundant, jointly constituting approximately 80% of the total gut flora.

While the species composition of the gut microbiota remained consistent across groups, we observed notable shifts in their relative abundances in **Figure 2B**. For instance, in the LEF group, there was a significant increase in Actinobacteria (rising from 4.6% in the RA1 group to 11.7% in the LEF group, $P < 0.01$) and a concurrent decrease in Bacteroidetes. Conversely, the introduction of Gui Zhi Paeoniae Zhi Mu Decoction (GSZD) led to an increase in Bacteroidetes (24.3%) and a marked decrease in Actinobacteria (7.9%).

The Bacteroidetes/Firmicutes ratio, a crucial indicator of gut health, showed the most substantial alteration in the LEGZ group, differing significantly from the other groups. This shift is particularly relevant as Bacteroidetes play a vital role in maintaining a healthy balance of gut microorganisms, while an overabundance of Firmicutes is often linked to detrimental health conditions. Thus, an increased Bacteroidetes/Firmicutes ratio could signal potential health benefits.

At the genus level in **Figure 2C**, we identified dominant genera such as Faecalibacterium,

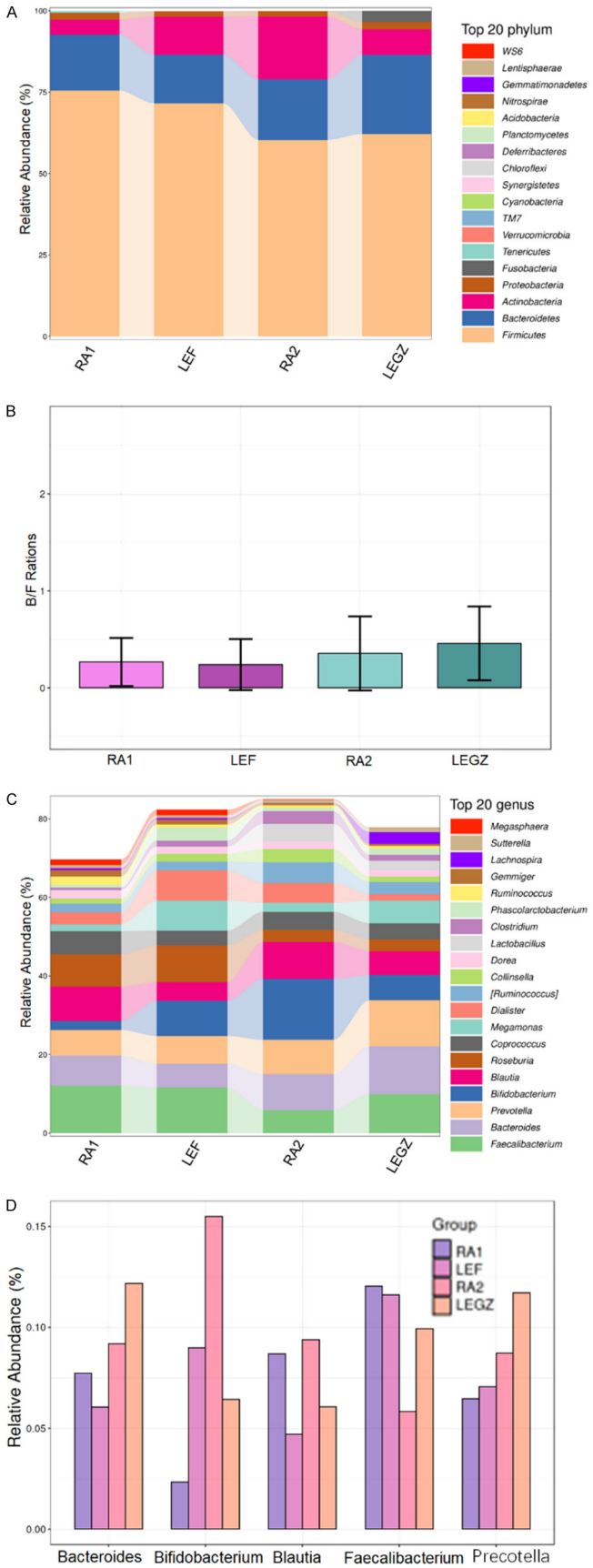


Figure 2. A. The abundance of the top 20 gut flora at the gate level. B. Bar graph showing the Bacteroidetes/Firmicutes (B/F) ratio variation in each group. C. The abundance of the top 20 gut flora at the genus level. D. Bar graphs showing the variation in genus level for each group with significant differences. RA1: pretreatment group for the LEF (Leflunomide) control group, RA2: pretreatment group for the LEGZ (Gui Zhi Shao Yao Zhi Mu Decoction combined with Leflunomide) experimental group.

Bacteroides, Prevotella, Bifidobacterium, and Blautia. The abundance of these genera was significantly altered by interventions with LEF or GSZD. LEF treatment notably increased the abundance of Bifidobacterium, Dialister, Megamonas, Phascolarctobacterium, and Collinsella, while reducing Bacteroides and Blautia. GSZD, on the other hand, decreased the abundance of these partial floras, which showed in the **Figure 2D**.

Lefse analysis of between-group differences

In our study, we employed the Linear Discriminant Analysis Effect Size (LEfSe) technique to discern significant differences in the gut microbiota composition between groups post-intervention with Leflunomide (LEF) and Gui Zhi Shao Yao Zhi Mu Decoction (GSZD). This analysis was pivotal in identifying specific bacterial taxa at the genus level that were differentially abundant, with a focus on those exhibiting a Linear Discriminant Analysis (LDA) score exceeding 2.0 and a *p*-value less than 0.05.

Our results revealed a total of 21 distinct intestinal markers between the RA1 and LEF groups, as illustrated in **Figure 3A** and **3B**. In the RA1 group, we observed an enrichment of 19 bacterial genera. These included key genera such as Streptococcus, Oscillospira, Ruminococcus, Enterococcus, Veillonella, Peptostreptococcus, Prevotella, Desulfovibrio, Synergistetes, Lactobacillus, Eggerthella, Lachnobacterium, Melissococcus, Christensenella, Pseudomonas, Dehalobacterium, Anaerofustis, Allobaculum,

and Oxalobacter. Conversely, in the LEF group, a distinctive presence of two genera, Collinsella and Campylobacter, was noted.

Further analysis, as shown in **Figure 3C** and **3D**, revealed that the RA2 group exhibited enrichment of four genera, namely Lactobacillus, Collinsella, Veillonella, and Streptococcus. In stark contrast, the LEGZ group showed a dominant presence of two genera, Lachnospira and Fusobacterium. These findings are indicative of the profound impact GSZD intervention has on the gut microbiota's compositional landscape at the genus level.

Symbiotic network for biota in the intestinal flora

In our study of the intestinal microbiota, we concentrated on the most abundant genera to map out their interrelationships. This analysis reveals a complex network of positive correlations, highlighting the intricate interactions within the gut ecosystem (**Figure 4**).

We identified significant overlaps in metabolic pathways between Blautia and Coprococcus, suggesting their collaborative role in fermenting dietary fibers and producing short-chain fatty acids. Similarly, genera pairings such as Paraprevotella with Parabacteroides, Bilophila with Phascolarctobacterium, and Enterococcus with Eubacterium share ecological niches, likely due to complementary metabolic functions that support their coexistence in the gut environment.

More complex symbiotic relationships are evident in clusters involving Besulfovibrio, Megasphaera, and Haemophilus; Bifidobacterium and Lactobacillus; as well as Adlercreutzia, Alistipes, Barnesiella, Oscillospira, and Ruminococcus. These clusters suggest a multi-genus collaboration, indicative of a resilient microbial community.

Additionally, our analysis revealed positive correlations among other genera, including Turicibacter, Veillonella, Actinomyces, Catenibacterium, Lachnobacterium, Weissella, Lactococcus, Streptococcus, Butyricicoccus, Dalister, Odoribacter, Mitsuokella, Butyricimonas, Bacteroides, Shigella, and Ruminococcus. Notably, Anaerostipes was found to correlate positively with Akkermansia, and Dorea with Clostridium, highlighting further complex inter-genus relationships within the gut microbiome.

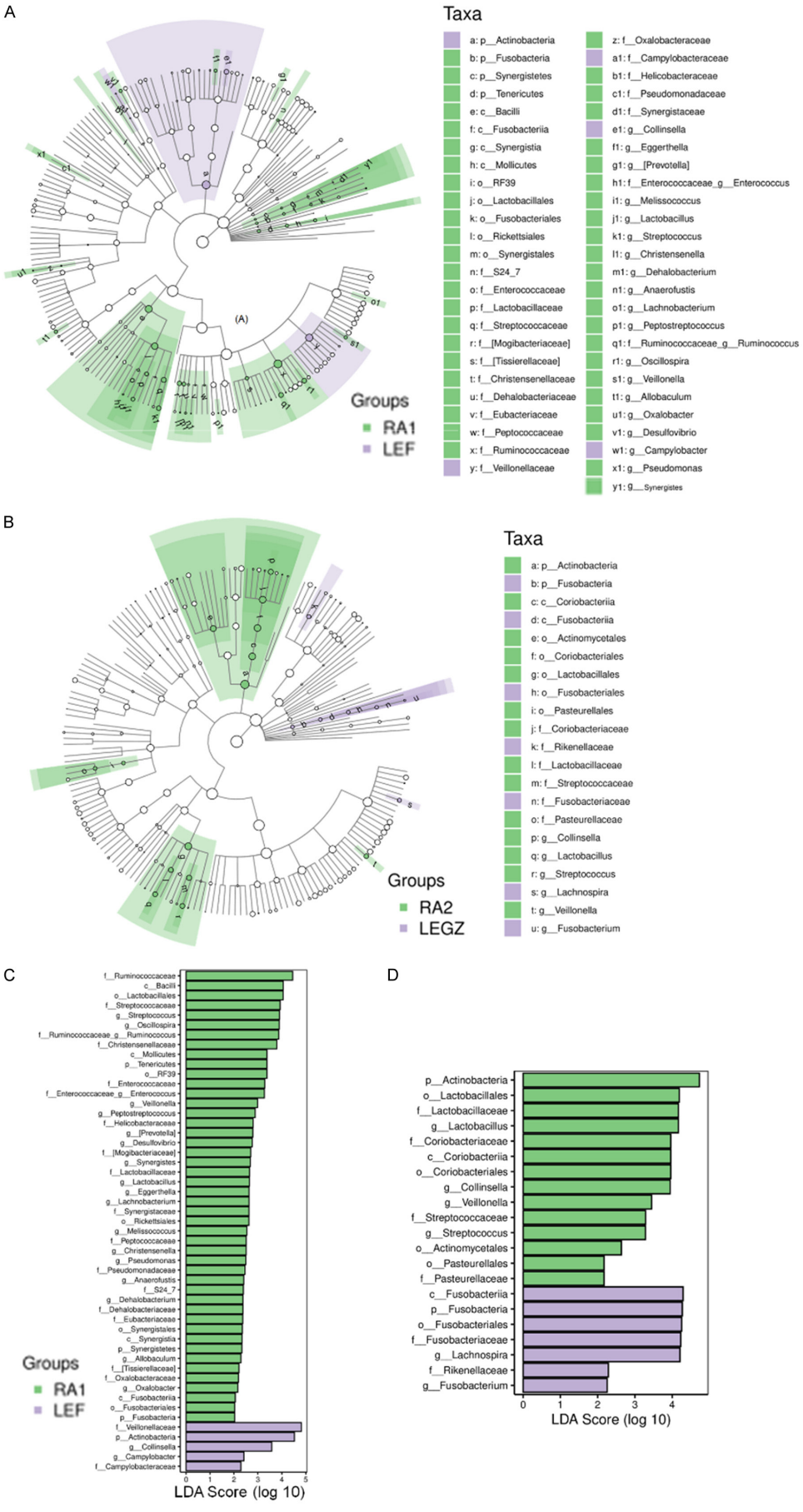
Expression of 16S functional genes and metabolic signaling pathways

We utilized PICRUST to predict the gut microbial function in each group, identifying 10 categorized 2-level KEGG ortholog groups in the dataset. Differential analysis of KEGG metabolic pathways revealed distinct variations in functional genes related to metabolism across different groups, helping us assess how the microbiota's metabolic profile adapts to environmental changes induced by various treatments.

The results, as shown in **Figure 5**, indicate that compared to the LEF group, the LEGZ group experienced an enrichment in six metabolic pathways. These pathways are involved in cell viability, transcription, degradation and metabolism of xenobiotics, metabolism of terpenoids and polyketides, other forms of hypometabolism, and carbohydrate metabolism. Conversely, there was a reduction in six other pathways in the LEGZ group, which are related to adenosine triphosphate (ATP) production, replication and repair, metabolism of other amino acids, metabolism of cofactors and vitamins, amino acid metabolism, and lipid metabolism.

Discussion

GSZD contains multiple active compounds that synergistically enhance its therapeutic effect in RA treatment [29]. In Taiwan, a significant portion of RA patients (76.4%) receive Chinese herbal medicines for treatment, with GSZD being the most commonly utilized formula [30]. Comparative studies indicate that TCM, specifically GSZD, may exhibit comparable or superior effectiveness and safety in treating RA compared to standard Western RA drugs. While GSZD showed a higher efficacy than placebo in some symptoms, only a few studies reported adverse events associated with GSZD, contrasting with several reports of serious adverse events in control groups treated with standard Western medicines or placebo [31]. Consequently, the critical role of GSZD in RA treatment has gained substantial attention, yet the underlying mechanisms of its therapeutic effects remain to be fully elucidated. Recent research has highlighted interactions between herbs and intestinal flora [32], but studies spe-


A
Taxa
a: p__Actinobacteria
b: p__Fusobacteria
c: p__Synergistetes
d: p__Tenericutes
e: c__Bacilli
f: c__Fusobacteriia
g: c__Synergistia
h: c__Mollicutes
i: o__RF39
j: o__Lactobacillales
k: o__Fusobacteriales
l: o__Rickettsiales
m: o__Synergistales
n: f__S24_7
o: f__Enterococcaceae
p: f__Lactobacillaceae
q: f__Streptococcaceae
r: f__[Mogibacteriaceae]
s: f__[Tissierellaceae]
t: f__Christensenellaceae
u: f__Dehalobacteriaceae
v: f__Eubacteriaceae
w: f__Peptococcaceae
x: f__Ruminococcaceae
y: f__Veillonellaceae
z: f__Oxalobacteraceae
a1: f__Campylobacteraceae
b1: f__Helicobacteraceae
c1: f__Pseudomonadaceae
d1: f__Synergistaceae
e1: g__Collinsella
f1: g__Eggerthella
g1: g__[Prevotella]
h1: f__Enterococcaceae_g__Enterococcus
i1: g__Melissococcus
j1: g__Lactobacillus
k1: g__Streptococcus
l1: g__Christensenella
m1: g__Dehalobacterium
n1: g__Anaerofustis
o1: g__Lachnobacterium
p1: g__Peptostreptococcus
q1: f__Ruminococcaceae_g__Ruminococcus
r1: g__Oscillospira
s1: g__Veillonella
t1: g__Allobaculum
u1: g__Oxalobacter
v1: g__Desulfovibrio
w1: g__Campylobacter
x1: g__Pseudomonas
y1: g__Synergistes
(A)
Groups
RA1
LEF
B
Taxa
a: p__Actinobacteria
b: p__Fusobacteria
c: c__Coriobacteriia
d: c__Fusobacteriia
e: o__Actinomycetales
f: o__Coriobacteriales
g: o__Lactobacillales
h: o__Fusobacteriales
i: o__Pasteurellales
j: f__Coriobacteriaceae
k: f__Rikenellaceae
l: f__Lactobacillaceae
m: f__Streptococcaceae
n: f__Fusobacteriaceae
o: f__Pasteurellaceae
p: g__Collinsella
q: g__Lactobacillus
r: g__Streptococcus
s: g__Lachnospira
t: g__Veillonella
u: g__Fusobacterium
Groups
RA2
LEGZ
C
f__Ruminococcaceae
c__Bacilli
o__Lactobacillales
f__Streptococcaceae
g__Streptococcus
g__Oscillospira
f__Ruminococcaceae_g__Ruminococcus
f__Christensenellaceae
c__Mollicutes
p__Tenericutes
o__RF39
f__Enterococcaceae
f__Enterococcaceae_g__Enterococcus
g__Veillonella
g__Peptostreptococcus
f__Helicobacteraceae
g__[Prevotella]
g__Desulfovibrio
f__[Mogibacteriaceae]
g__Synergistes
f__Lactobacillaceae
g__Lactobacillus
g__Eggerthella
g__Lachnobacterium
f__Synergistaceae
o__Rickettsiales
g__Melissococcus
f__Peptococcaceae
g__Christensenella
g__Pseudomonas
f__Pseudomonadaceae
g__Anaerofustis
f__S24_7
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f__Dehalobacteriaceae
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f__Oxalobacteraceae
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c__Fusobacteriia
o__Fusobacteriales
p__Fusobacteria
f__Veillonellaceae
p__Actinobacteria
g__Collinsella
g__Campylobacter
f__Campylobacteraceae
Groups
RA1
LEF
0 1 2 3 4 5
LDA Score (log 10)
D
p__Actinobacteria
o__Lactobacillales
f__Lactobacillaceae
g__Lactobacillus
f__Coriobacteriaceae
c__Coriobacteriia
o__Coriobacteriales
g__Collinsella
g__Veillonella
f__Streptococcaceae
g__Streptococcus
o__Actinomycetales
o__Pasteurellales
f__Pasteurellaceae
c__Fusobacteriia
p__Fusobacteria
o__Fusobacteriales
f__Fusobacteriaceae
g__Lachnospira
f__Rikenellaceae
g__Fusobacterium
0 1 2 3 4
LDA Score (log 10)

Figure 3. A, B. Cladogram drawn with the Lefse method shows the fecal microorganisms' phylogenetic distribution associated with the four groups. C, D. LDA scores show significant differences in gut flora between groups. Larger LDA scores represent a more significant effect of species abundance on group differences. RA1: pretreatment group for the LEF (Leflunomide) control group, RA2: pretreatment group for the LEGZ (Gui Zhi Shao Yao Zhi Mu Decoction combined with Leflunomide) experimental group.

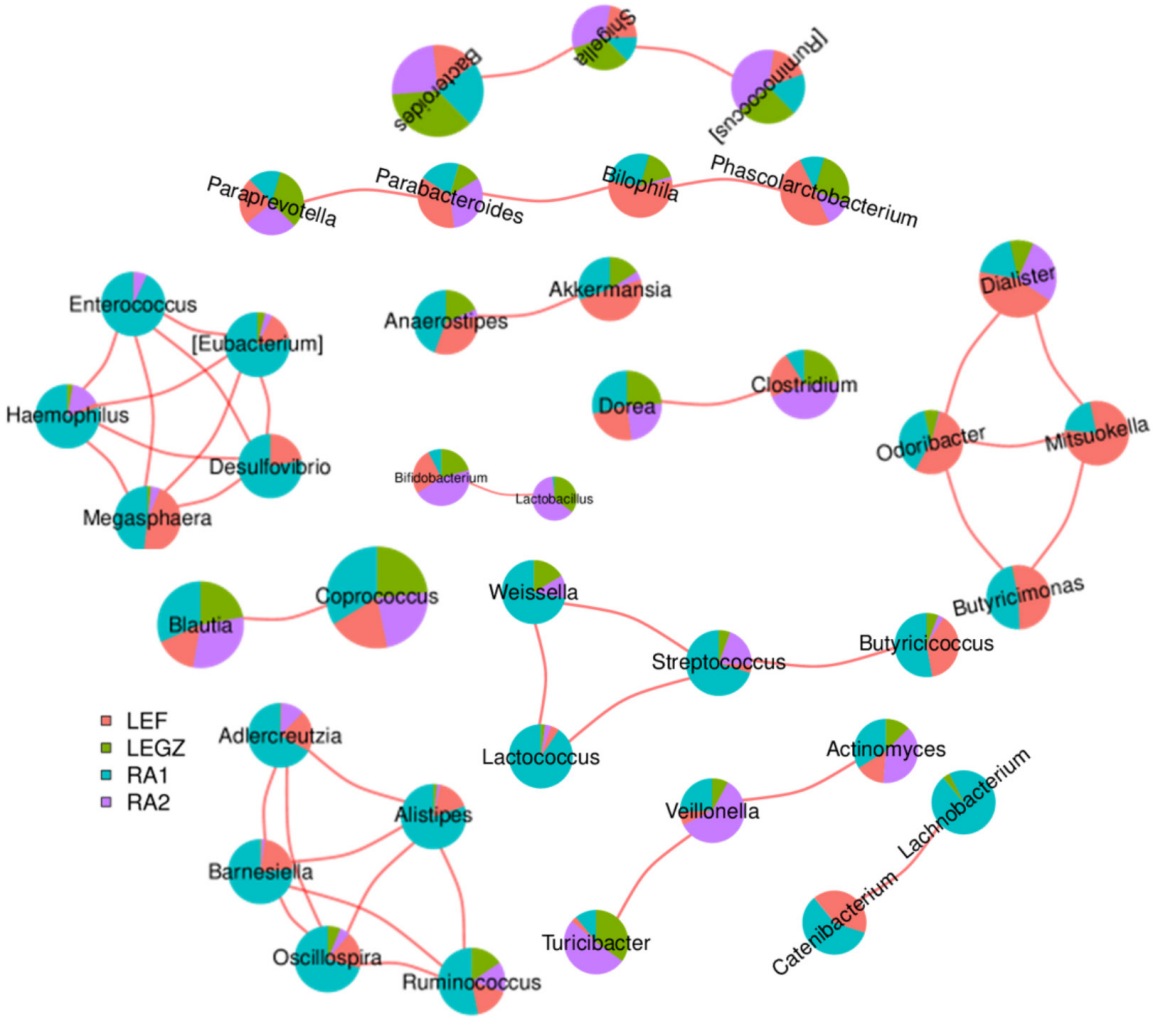

Figure 4. Analysis of association network for each group. RA1: pretreatment group for the LEF (Leflunomide) control group, RA2: pretreatment group for the LEGZ (Gui Zhi Shao Yao Zhi Mu Decoction combined with Leflunomide) experimental group.

cifically investigating the effect of GSZD on intestinal flora in RA treatment are limited and warrant further exploration. In our study, the preference for Leflunomide over Methotrexate, attributed to its antitumor effects, influenced its selection as the control group, with the combination of GSZD and Leflunomide forming the test group [33]. We analyzed the correlation coefficients between clinical data of RA1, LEF, RA2, and LEGZ groups to assess RA severity. Post-treatment, significant reductions in RA severity indices were observed in both the LEF and LEGZ groups, with a more pronounced effect in the LEGZ group, suggesting GSZD's potential in relieving RA pain. Clinical indicators such as ESR, CRP, and RF, which are related to metabolic processes like glucose metabolism [34], protein expression [35], phenylalanine metabolism [36], and the immune system [37], indicate that GSZD may influence these processes and the immune system through regulation of the intestinal flora.

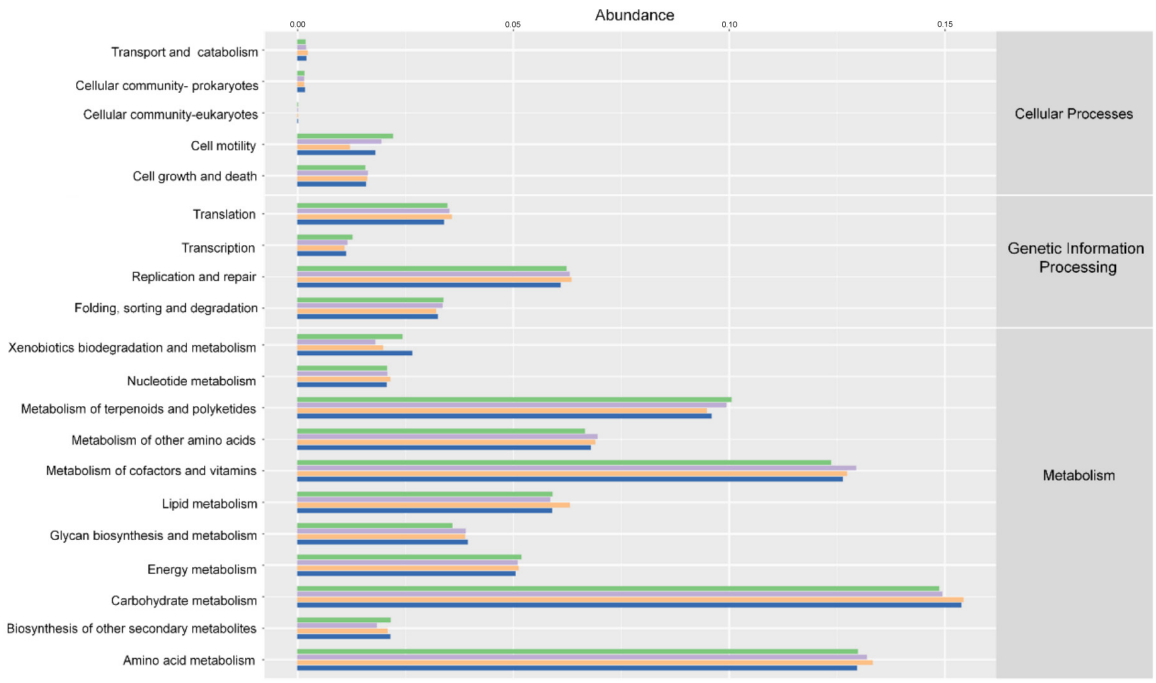

Figure 5. Analysis of KEGG pathway for each group of intestinal flora. RA1: pretreatment group for the LEF (Leflunomide) control group, RA2: pretreatment group for the LEGZ (Gui Zhi Shao Yao Zhi Mu Decoction combined with Leflunomide) experimental group.

Our 16S rDNA sequence analysis did not show significant changes in alpha and beta diversity among the different treatments. At the phylum and genus levels, we observed shifts in species abundance. Elevated levels of Actinobacteria in RA patients [38] were reduced following GSZD treatment, suggesting a regulatory effect on intestinal microecology. The role of Bacteroidetes in polysaccharide metabolism [39] and calorie absorption [40] is noteworthy, as is their involvement in metabolizing phenylalanine into phenylpropanoid acid [41] and modulating the immune response through TLR4 receptors [42]. Overgrowth of Firmicutes is linked to metabolic disorders like obesity [43] and type 2 diabetes [44], and their production of short-chain fatty acids (SCFA) and other metabolites [45], is crucial for health. The observed increase in Bacteroidetes relative to Firmicutes in patients treated with GSZD could be a mechanism by which GSZD treats RA, improving metabolic functions and enhancing intestinal barrier integrity [46].

At the genus level, significant differences in the abundance of Faecalibacterium, Bacteroides, Prevotella, and Bifidobacterium were noted between groups. The increased abundance of Faecalibacterium, Bacteroides, and Prevotella, which regulate the immune system through Toll-like receptors (TLR) [47-49], suggests a stabilizing effect on the intestinal environment. Although the regulatory receptors of Bifidobacterium are not well studied, its role in immune stimulation and barrier effects is recognized [50]. Our results align with increased abundance of these genera in the LEGZ group, with a notable low level of Bifidobacterium, potentially linked to the drug components' effects. GSZD has been shown to alleviate RA symptoms by modulating the HDAC1-HSP-90AA1-NFKB2-IKBKB-TNF-α signaling axis, with 77 significant components identified out of 165 components in GSZD [51], suggesting its complex composition and broad impact on the intestinal flora. Furthermore, ecological dysbiosis in RA patients, characterized by the expansion of rare and harmful lineages, was addressed in our study [38]. Lefse analysis revealed a contraction of harmful genera (such as Lactobacillus, Veillonella, and Streptococcus) and expansion of beneficial genera in both LEF and LEGZ groups. Specifically, the LEGZ-enriched Lachnospira, an anti-inflammatory bacterium [52], is involved in carbohydrate metabolism and promotes intestinal health by

producing SCFA [53]. These results indicate that GSZD intervention impacts the intestinal flora's composition at the genus level.

Through symbiotic overlap network analysis of biota, correlations between certain genera were identified. The positive correlation between these genera suggests similar ecological functions or synergistic effects beneficial for intestinal health and homeostasis. Bifidobacterium [50] and Lactobacillus [53] are known probiotics, while Blautia and Coprococcus [54] play a role in SCFA synthesis and intestinal health. However, further research is needed to fully understand microbial community interactions.

KEGG analysis results indicate that the enriched pathways in the LEGZ group, compared to the LEF group, may contribute more significantly to cellular metabolic viability, response to environmental changes, and synthesis of bioactive substances. The decreased pathways in the LEGZ group might reflect GSZD's regulation of abnormal metabolism in the intestine. In vitro experiments demonstrated GSZD's ability to reduce inflammatory factor release in LPS-stimulated RAW 264.7 cells [55], disrupt the RA disease dysfunction module, and restore *in vivo* homeostasis [56]. These results highlight significant metabolic pathway differences between the intestinal flora of the LEF and LEGZ groups, possibly due to GSZD's complex composition and broader impact compared to LEF. Further in-depth studies are required to elucidate these mechanisms. However, these individuals are susceptible to developing cancer as a result of the immunosuppressive treatment as well as degradation of muscular and skeletal tissues, which necessitates accurate prediction using sophisticated methods [57, 58]. The salient aspect to consider is that the implementation of alternative methodologies in conjunction with conventional medical interventions can potentially mitigate numerous adverse effects associated with RA [59]. Our study, while providing valuable insights, is not without its limitations, which are important to consider for a comprehensive understanding of our findings. The most notable limitation is the relatively small sample size of 48 participants, with 42 ultimately included in the final analysis. This was primarily due to the stringent selection criteria aimed at ensuring a homogenous participant pool, which, while beneficial for reducing variability, inevitably limited the number of eligible participants. Additionally, logistical constraints such as resource availability and time factors played a significant role in determining the feasible number of participants. While these limitations might affect the generalizability of our findings, they also reflect the realistic challenges often encountered in specialized research areas. We have endeavored to mitigate these limitations through robust methodological approaches and thorough statistical analysis. Nonetheless, we acknowledge that these factors necessitate cautious interpretation of the results and underscore the need for further studies with larger and more diverse populations to validate and extend our findings. The insights gained from this study lay the groundwork for future research, highlighting critical areas for exploration and providing a basis for more extensive investigations in this field.

Conclusion

Our study provides compelling evidence that GSZD can significantly modulate both the composition and metabolic functionality of the gut microbiota in patients with RA. These findings enrich our understanding of RA and unveil potential mechanisms through which GSZD influences the gut microbiota-host metabolic equilibrium in RA. This research contributes to advancing the development of more targeted and effective therapies for RA, blending traditional Chinese medicine with modern pharmacological approaches.

Acknowledgements

This work was supported by Shunyi District Special Development Scientific Research Special Shunyi Special Research on Health Development (WSJKFZKYZX-2019-Q-02).

Disclosure of conflict of interest

None.

Ethics statement

This study was conducted in strict accordance with the ethical principles for medical research involving human subjects as outlined in the Declaration of Helsinki. The study protocol was reviewed and approved by the Institutional

Review Board (IRB) of Shunyi Hospital, WSJKFZKYZX-2019-Q-02. Prior to participation, all participants were provided with comprehensive information about the study objectives, procedures, potential risks, and benefits. Informed consent was obtained from each participant or their legal guardian. Participants were assured of confidentiality and the right to withdraw from the study at any time without any consequences to their medical care. All data were anonymized to maintain participant privacy. The approval report and related ethical clearance documents are provided as supplementary files to this manuscript.

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Table S1. The Sequencing Result

SampleID	Input	Non-chimeric
RA1	100030	49133
RA2	96584	55369
RA3	88540	49121
RA4	80805	49746
RA5	73957	48215
RA6	68867	40126
RA7	61967	37193
RA8	74508	46156
RA9	75325	46417
RA10	105145	60706
RA11	86792	58389
RA12	84043	57411
RA13	92977	71923
RA14	99122	75347
RA15	108716	97271
RA16	142204	74528
RA17	140340	75234
RA18	135296	76897
RA19	88610	56544
RA20	101358	62847
RA21	120590	85482
LEF1	95949	55072
LEF2	113426	65975
LEF3	74168	48007
LEF4	125818	86498
LEF5	120967	89729
LEF6	132179	83183
LEF7	107096	60796
LEF8	79010	44936
LEF9	82893	49395
LEF10	106529	68683
LEF11	101403	58755
LEF12	101500	60000
LEF13	111550	100530
LEF14	93603	63623
LEF15	114344	78046
LEF16	129294	79387
LEF17	125952	75793
LEF18	89737	54436
LEF19	104949	62727
LEF20	99265	63988
LEF21	83920	54931
RA_1	98486	69408
RA_2	77358	69903
RA_3	93609	74097
RA_4	135554	117430
RA_5	151476	103659

RA_6	147810	101686
RA_7	107706	60836
RA_8	77348	49390
RA_9	89855	57789
RA_10	76555	52607
RA_11	68543	47074
RA_12	77262	56719
RA_13	93713	75281
RA_14	83777	60285
RA_15	87268	75778
RA_16	133918	79490
RA_17	71269	40156
RA_18	87238	50461
RA_19	87143	46821
RA_20	85278	52497
RA_21	95969	64084
LEGZ1	151620	104582
LEGZ2	151918	102809
LEGZ3	146755	109461
LEGZ4	196611	133763
LEGZ5	183466	154999
LEGZ6	190295	167584
LEGZ7	143787	99740
LEGZ8	137734	103259
LEGZ9	206517	183217
LEGZ10	205680	122010
LEGZ11	198905	141937
LEGZ12	183589	150504
LEGZ13	164411	145705
LEGZ14	195540	162808
LEGZ15	180680	141514
LEGZ16	145805	84947
LEGZ17	124053	76989
LEGZ18	183535	107267
LEGZ19	215508	183226
LEGZ20	205964	113584
LEGZ21	219507	128147
average	118789.8	80976.76

Table S2. OTU Coverage of All Gr

Sample	Goods_coverage
RA1	0.990249
RA2	0.991169
RA3	0.991787
RA4	0.994347
RA5	0.995365
RA6	0.995753
RA7	0.997251
RA8	0.995719
RA9	0.99503
RA10	0.991255
RA11	0.994264
RA12	0.995027
RA13	0.998628
RA14	0.996756
RA15	0.998117
RA16	0.982639
RA17	0.983416
RA18	0.991389
RA19	0.995388
RA20	0.99465
RA21	0.995513
LEF1	0.987237
LEF2	0.986145
LEF3	0.992647
LEF4	0.996308
LEF5	0.997099
LEF6	0.995679
LEF7	0.990515
LEF8	0.993073
LEF9	0.993267
LEF10	0.99207
LEF11	0.989252
LEF12	0.991387
LEF13	0.998471
LEF14	0.996139
LEF15	0.995039
LEF16	0.989409
LEF17	0.989386
LEF18	0.992304
LEF19	0.992084
LEF20	0.993667
LEF21	0.994599
RA_1	0.998631
RA_2	0.999394
RA_3	0.99892
RA_4	0.998954
RA_5	0.996202

RA_6	0.996416
RA_7	0.991201
RA_8	0.99495
RA_9	0.994562
RA_10	0.997128
RA_11	0.997188
RA_12	0.997085
RA_13	0.999171
RA_14	0.998728
RA_15	0.99962
RA_16	0.989132
RA_17	0.994825
RA_18	0.992213
RA_19	0.995193
RA_20	0.995719
RA_21	0.995239
LEGZ1	0.998122
LEGZ2	0.997971
LEGZ3	0.99824
LEGZ4	0.994562
LEGZ5	0.99814
LEGZ6	0.998414
LEGZ7	0.995873
LEGZ8	0.995833
LEGZ9	0.998565
LEGZ10	0.995328
LEGZ11	0.997382
LEGZ12	0.998177
LEGZ13	0.999251
LEGZ14	0.998783
LEGZ15	0.998322
LEGZ16	0.988735
LEGZ17	0.99299
LEGZ18	0.984839
LEGZ19	0.998657
LEGZ20	0.994819
LEGZ21	0.992853