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Original Article

Changes of Intestinal Microecology in Patients with Primary Sjogren's Syndrome after Therapy of Yangyin Yiqi Huoxue Recipe (养阴益气活血方)*

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ABSTRACT **Objective:** To explore the change of intestinal microecology in patients with primary Sjogren's syndrome (pSS) and correlation with disease activity, and also discuss the therapy effect of Yangyin Yiqi Huoxue Recipe (养阴益气活血方, YYHD). **Methods:** Sixteen pSS patients were enrolled in the present study, who received 3-month treatment of YYHR, 200 mL orally twice daily. Their pre-and post-test ESSDAI scores, erythrocyte sedimentation rate (ESR) and serum immunoglobulin G (IgG) levels were measured respectively. The 16SrDNA metagenomic sequencing was used to detect and analyze the abundance and diversity of intestinal bacteria flora and the proportion of bacteria at the levels of phylum, family, and genus, in comparision with those of 6 healthy subjects in the control group. **Results:** The abundance and diversity of intestinal bacteria flora in pSS patients were lower than those of healthy subjects (P<0.05). After the treatment with YYHD, patients' ESSDAI score and levels of IgG and ESR have decreased significantly (P<0.05). At the phylum level, the proportions of *Actinobacteria*, *Firmicutes*, *Fusobacteria* and *Proteobacteria* have reduced sharply, while the proportions of *Bacteroidetes*, *Teneriquetes* and *Candidate-division*-TM7 have increased significantly by treatment (all P<0.05). At the classification level, such treatment has caused a significant decrease in the proportions of *Bacteroidaceae*, *Ruminococcaceae*, *Veillonellaceae*, and *Enterobacteriacea* (all P<0.05), but a significant increase in the proportion of *Lachnospiraceae* (P<0.05). At the genus level, the treatment has significantly decreased the proportions of *Bifidobacterium*, *Bacteroides*, *Escherichia-Shigella*, *Faecalibacterium* and *Prevotella* (all P<0.05), but significantly increased the proportion of *Clostridia* (P<0.05), close to the levels of healthy subjects (P>0.05). **Conclusions:** There exists an imbalance of intestinal microecology in pSS patients, which can be improved through the treatment with YYHD. Besides, such treatment can also improve the disease activity and adjust the diversity of intestinal bacteria flora, the composition and the abundance of intestinal flora.

KEYWORDS primary Sjogren's syndrome, intestinal microecology, Yangyin Yiqi Huoxue Recipe, disease activity, 16SrDNA metagenomic sequencing

Primary Sjogren's syndrome (pSS) is a rheumatoid immune disease mainly invading the exocrine glands, which is commonly found in women aged 40 to 50 years. Patients often have obvious symptoms such as a dry mouth and both dry eyes, which severely affects their quality of life.[1] So far, there has been no effecive treatment. The clinical therapy mainly includes symptomatic treatment and alternative treatment, but pSS is easy to relapse.[2] Chinese medicine (CM) treatment has great advantages in improving patients' symptoms, controlling the disease and improving the quality of life, with long-term stable effects.[3]

In our previous clinical studies, Yangyin Yiqi Huoxue Recipe (养阴益气活血方, YYHR) has shown a curative effect on pSS, not only relieving the disease, but also regulating the Th1/Th2 immune balance and reproductive hormone-endocrine-immune function, which helps improve the quality of life.[4,5] Recent studies have indicated that changes of the body's

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microbial flora are closely related to the occurrence of autoimmune diseases, and microbial flora is one of the factors affecting the body's immune system and the progression of autoimmune diseases.[6] The microbiome plays a certain role in maintaining homeostasis of the immune system. These studies have also shown that the intestinal microecology underwent certain changes in pSS patients.[6,7] Therefore, we carried out this study to explore how YYHR regulates the intestinal microecology in pSS patients.

METHODS

Inclusion Criteria

The inclusion criteria were as follows: (1) according with the pSS classification standard launched by the American College Rheumatology (ACR) along with the European League Against Rheumatism criteria (EULAR) in 2016;[8] (2) diagnosed as qi and yin deficiency and blood stasis syndrome;[9] (3) no complications like lymphoma, diabetes, severe liver and kidney disease, metabolic disease or infectious disease; (4) no history of intestinal disease and no special dietary habits; (5) no use of antibiotics, probiotics or corticosteroid within 2 months; and (6) all patients with good compliance signed the informed consent form.

Exclusion Criteria

The exclusion criteria were as follows: (1) patients with other rheumatic immune diseases; (2) patients subjected to serious diseases such as heart, brain and hematopoietic system; (3) pregnant or lactating women, and mental patients; and (4) poor dependency.

Subjects

Sixteen female outpatients and inpatients were enrolled in the First Affiliated Hospital, School of Medicine, Zhejiang University from October 2016 through December 2017. These patients were 25–65 years old, with an average age of 38.4 ± 24.5; the duration of disease was 1–8.6 years with an average of 4.6 ± 1.9 years.

In addition, six healthy adult women who have physical examination in The First Affiliated Hospital, College of Medicine, Zhejiang University were selected as healthy subjects. Their ages ranged from 28 to 62, with an average of 41.7 ± 25.2 years. These healthy subjects did not have any special dietary habits or take antibiotics within 2 months. This study has been approved by the Ethics committee of The First Affiliated Hospital, College of Medicine, Zhejiang University [Reference Number: (2014) Scientific research quick approval No. 009]

Treatment

All pSS patients took YYHD, once enrolled. YYHD was composed of *Radix Pseudostellariae* 24 g, *Radix paeoniae alba* 18 g, *Rhizoma Polygonati* 18 g, *Fructus Ligustri Lucidi* 15 g, *Rhizoma Dioscoreae* 30 g, *Fructus Schisandrae* 10 g, *Fructus Mume* 15 g, and *Rhizoma Rhodiolae sachalinesis* 15 g, *glabrous sarcandra* herb 12 g. For patients with joint pains, *Clematis chinensis* 15 g, *Siegesbechia herb* 15 g, and *futokadsura stem* 30 g were added to the formula. For patients with obvious mouth dryness, *Trichosanthes root* 15 g and *Radix Ophiopogonis* 15 g were added. For patients with obvious eye dryness, *Medlar* 15 g and *Flos Buddlejae* 12 g were added. For patients with fatigue, *Radix astragali* 15 g and *Herba Agrimoniae* 15 g were added. These Chinese herbs were made into 400 mL decoction by the Traditional Chinese Medicine Preparation Room of the First Affiliated Hospital, College of Medicine, Zhejiang University before being divided into 2 vacuum bags (200 mL each). Patients took 2 bags of the formulae each day, 30 min after breakfast and dinner for 3 consecutive months as one course of treatment. Disease evaluation and indicators testing were performed before and after the treatment. Besides, the healthy control group was only tested when subjects were enrolled.

Specimen Collection

Serum Specimen

Subjects were fasted 12 h overnight before the blood collection. Elbow vein blood was extracted in the early morning. Serum was got by centrifuge, installed in EP tubes and stored in the –80℃ container for use.

Feces specimen

Fresh feces specimens (300 mg) were collected in sterile boxes at 6:00–8:00 am. Bacteria flora inoculation was conducted in 30 min. The feces specimens were stored in the –80 ℃ container for use.

Analysis of pSS Disease Activity Degree

ESSDAI Score

EULAR SS Disease Activity Index (ESSDAI) score is a commonly used disease activity index. It was proposed by EULAR, and applied to systemically evaluate pSS disease activity from 12 systemic lesions,

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including general symptom, lymph nodes, exocrine glands, joints, skin, lungs, kidneys, muscles, central nervous system, peripheral nerves, blood systems and serological manifestations.[10] Disease activity score is the sum of each system score. ESSDAI score was evaluated before and after treatment.

Disease Activity Indicators Measurement

Serum erythrocyte sedimentation rate (ESR) and serum immunoglobulin G (IgG) were measured by methods of Westergren method and radioimmunology, respectively.

Testing and Analysis of Intestinal Microecology

Extraction of Total Feces Genome DNA

A feces specimen (200 mg) of both groups was placed in a 2-mL centrifuge tube. The total DNA of intestinal flora was extracted using Omega's DNA extraction kit [Tiangen Biotech (Beijing) Co., Ltd., China] in strict accordance with corresponding instructions and was stored in –20 ℃ for use.

PCR Amplification of Total DNA in Feces Flora

The total DNA of the fecal flora was placed at the PCR instrument. The first round PCR amplification products were obtained after a series of steps: denaturation, annealing and extension. PCR products (2 μL) were subjected to electrophoresis in 2% agarose gel to confirm the target product fragments. Then the PCR products were purified using the 1 × volume AMPpure XP beads. The purified PCR products went through the second amplification. The primer information is illustrated in Table 1.

Library Purification

The target fragments after amplification went through the electrophoresis with 2% agarose gel, and then gel extraction according to the procedures of QIAquick Gel extraction kit (QIAGEN, Germany).

Quality Assessment and Quantification

Qubit fluorometer (Invitrogen-Q32866, Life Technologies, USA) was used to measure the DNA concentration of the library. The DNA with concentration > 1.0 ng/L was considered qualified. Qseq100 DNA analyzer (BiOptic Inc, Taiwan, China) was used to measure the DNA length distribution of the library. The DNA, which met requirements such as the length of the target fragment, single peak, without joint peak or large peak, was considered eligible. The KAPA library quantification kit was used to quantify the molar concentration of the DNA of the library, which acted as the standard for library mixing.

Miseq Sequencing and Sequence Analysis of V3-V4 Variable Region of Fecal Genome 16SrDNA

After mixing and degeneration, the library (Illumina/Universal, KAPA Biosystems, USA) was added to the Illumina Miseq sequencing platform (MiSeq System, Illumina, USA) for high throughput sequencing according to the manual instruction. The two ends of the library were sequenced (i.e. Paired-end, PE), so each specimen generated two data files: reads 1 (R1) and reads 2 (R2).

Bioinformatics Analysis

Bacterial Species Analysis and Annotation

All the original data were subjected to sequence split joint and filter to get clean data. Then, based on valid data, the operational taxonomic units (OUTs) clustering and species classification annotation were performed to obtain OTU data of each specimen and grouping and

Table 1. Primer Information for Total DNA in Feces Flora

Amplicon name	Primer name	Primer sequence (5'–3')	Amplification region	Product length (bp)	Annealing temperature (℃)
Bacteria 16S	B341F	CCTACGGGNGGCWGCAG	V3-V4	450	55
	B785R	GACTACHVGGGTATCTAATCC			
Archaea 16S	A349F	GYGCASCAGKCGMGAAW	V3-V4	420	55
	A806R	GGACTACVSGGGTATCTAAT			
Fungus ITS	ITS-3	GATGAAGAACGYAGYRAA	ITS2	350	53
	ITS-4	TCCTCCGCTTATTGATATGC			
Fungus 18S	EF4	GGAAGGGRTGTATTTATTAG	–	380	55
	NS2	GGCTGCTGGCACCAGACTTGC			

Notes: There is a MISeq platform common sequencing connector at the 5' end of the primer sequence, which binds to the second round of primer at the second round of amplification. In the primer sequences, the simple bound bases refer to as follows: R: A/G; Y: C/T; M: A/C; K: G/T; S: G/C; W: A/T; H: A/T/C; B: G/T/C; V: G/A/C; D: G/A/T; N: A/G/C/T.

classified pedigree. OTU data was further analyzed in terms of the abundance and diversity of bacteria flora. Cluster analysis and PCoA were performed based on the results of OTU and species annotation to elucidate the taxonomy difference of bacteria flora among specimens and among different groups, and to elucidate the bacteria species closely related to pSS disease. The descriptive indices of microorganism diversity more commonly used Simpson—the Simpson index, and Shannon—the Shannon index. The descriptive indices of the flora abundance were the Chao1 estimator and the Ace estimator reaction.

Bacterial Species Clustered

The sequence was clustered into OTU according to the sequence similarity. The similarity threshold was set at 97%, that is, a sequence with the similarity higher than 97% was clustered as an OTU. The singleton sequence was discarded. The OTU sequence was systematically classified into 6 levels based on Bergey's taxonomy, which were kingdom, phylum, class, order, family and genus. The default threshold was 80%. Below this value was attributed to unclassified.

Alpha Diversity of Bacterial Species

Alpha diversity was used to compare the species diversity of intestinal flora between different groups. Alpha diversity reflects the species diversity within a single sample. The indices include observed species index, Chao index, ACE index, Shannon index and Simpson index. The greater the first four indices and the smaller the last index in one sample, the richer the species diversity of the microorganisms in this sample.

Dilution Curves and Shannon-Wiener Curves

The dilution curve is used to estimate the flora abundance with 97% similarity. The curve can be used to compare the abundance of intestinal flora between patients and healthy subjects, as well as to explain the rationality of sample sequence. When the curve trends to flat in the figure, it means sequencing data is reasonable. The Shannon-Wiener curve is constructed based on flora diversity index of samples at different sequencing depths. It is an indicator to describe the extent of microbial diversity. The curve reflects the abundance and uniformity of the samples. The horizontal axis positively represents OTU abundance. The bigger the horizontal axis is, the higher OTU abundance is. The flatter the curve is, the more uniform OTU distribution is.

Bacterial Species Beta Diversity

Beta diversity is an ecological concept that refers to the difference of species composition between different groups. Principal component analysis (PCA) is a sequencing analysis based on matrix composed of original species. PCA analysis is usually used in 16S data analysis.

Statistical Analysis

The Linear Discriminant Analysis (LDA) Effect Size is a software that performs linear discrimination analysis between different groups to seek flora or species with significant difference. This software can be used to statistically analyze the composition similarity and difference of microbial flora among groups.

Bacterial Species Linear Analysis

All intestinal high throughput sequencing data information was clustered into OTU using USEARCH[11] according to sequence similarity. Similarity threshold was set at 97%. A sequence with a similarity of > 97% was clustered into one OTU. Diversity analysis was performed using mothur software on the basis of OTU.[12] That is, the bacteria abundance (Ace index, Chao index) and flora diversity (Simpson index, Shannon index) of all the fecal specimens were calculated. Metastats[13] was used to test the difference of relative abundance of species between groups. The rest data were analyzed by SPSS 19.0 statistical software, represented by $\bar{x} \pm s$. The data comparison of intergroups was performed by *t*-test. $P<0.05$ was considered significantly different.

RESULTS

General Condition

There was no significant age difference between the healthy control group and pSS group. Sixteen pSS patients and six healthy adults enrolled in the present study have shown good dependence, without any loss or withdrawal throughout the process.

Effect of YYHR on Disease Activity of pSS

The ESSDAI scores and the levels of ESR and IgG were significantly higher in pSS patients before treatment compared to those in healthy subjects ($P<0.05$). After the treatment with YYHD, compared with before treatment, all indices were decreased significantly ($P<0.05$). There was no significant

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Table 2. Comparison of pSS Disease Activity before and after Treatment ($\bar{x} \pm s$)

Group	ESSDAI scores	ESR (mm/h)	IgG (mg/mL)
Healthy control			
(6 cases)	0	6.8 ± 2.1	926.7 ± 179.3
pSS			
BT (16 cases)	3.38 ± 1.75*	33.0 ± 12.5*	2471.9 ± 424.8*
AT (16 cases)	1.81 ± 1.42*△	15.1 ± 4.0△	1723.1 ± 354.7*△

Notes: *P<0.05 *vs*. healthy control group; △P<0.05 *vs*. before treatment in the same group; BT: before treatment, AT: after treatment.

difference in ESR between patients after treatment and healthy subjects (P>0.05, Table 2).

Bacteria Distribution at Different Classification Levels

Through the species annotation analysis, the OTU data in 38 samples (16 patients before and after treatment, 6 healthy subjects) were classified. The components of each sample were stated and counted at the level of the phylum, class, order, family, genus, and species. Given that the levels of the class and order did not reflect the difference when compared to the phylum, as well as the great number of varieties at the species level, phylum, family and genus were chosen as representatives for further analysis.

Distribution of Intestinal Flora at Phylum Level

In this study, 15 phylums were identified from all sequences from both the healthy subjects and pSS patients. Some sequences were still unclassified. Among the 15 phylums, the highest proportions were *Fimicutes* and *Bacteroidetes*, followed by *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Candidate*_division_*TM7*, *Spirochaetae*, *Verrucomicrobia*, *Tenericumtes*, *Cyanobacteria*, *Synergistetes*, *Elusimicrobia*, *Lentisphaerae*, *Deferribacteres* and *Euryarchaeota*. *Euryarchaeota* was the only species of *Archaeans* species found in healthy subjects, but not in pSS patients (Figure 1A).

Distribution of Intestinal Flora at Genus Level

At the genus level, 40 species and some unclassified ones were identified from all sequences. The most common genus included *Bacteroides*, *Faecalibacterium*, *Prevotella*, *Escherichia-Shigella*, *Incertae-Sedis*, *Roseburia*, *Parabacteroides* and *Allisonella*. Among them, *Faecalibacteriuma* was not detected in healthy subjects (Figure 1B).

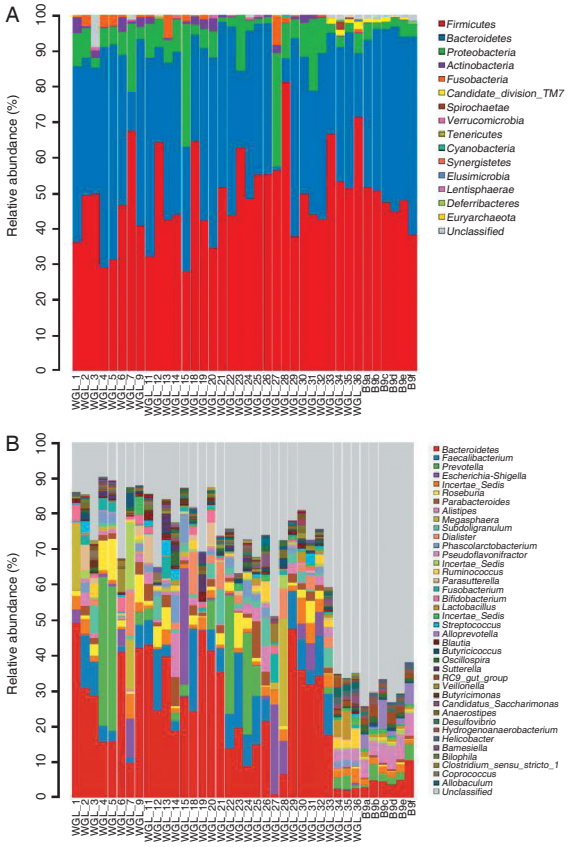

Figure 1. Distribution of Intestinal Flora at Phylum Level (A) and Genus Level (B)

Comparison of Alpha Diversity of Intestinal Flora

Analysis of Intestinal Bacteria Diversity

Shannon index, Ace index, Chao index and Sobs index of the intestinal flora in the pSS group before treatment were lower than those in the healthy control group with statistically significant (P<0.05). These indices increased in varying degrees by treatment, with differences statistically significant compared to those before treatment (P<0.05) except for the Simpson index. The Simpson index of the intestinal flora in the pSS group before treatment was higher than those of the healthy control group (P<0.05). Treatment decreased the Simpson index, but the decrease was not statistically significant (P>0.05, Table 3).

Dilution Curve and Shannon-Wiener Curve of Intestinal Bacteria Flora

The sequencing results in this study were sufficient to reflect the diversity and uniform distribution of microorganisms (Figure 2).

Analysis of Beta Diversity of Intestinal Flora

Through PCA analysis of different sample individuals, it was found that the distance was far in the

Table 3. Analysis of Intestinal Bacteria Diversity ($\bar{x} \pm s$)

Group	Time	Case	Shannon index	Simpson index	Ace index	Chao index	Sobs index
Healthy control		6	4.80 ± 0.13	0.02 ± 0.00	902.48 ± 74.71	892.32 ± 70.22	746.50 ± 75.50
pSS	Before-treat.	16	3.23 ± 0.52*	0.10 ± 0.07*	355.63 ± 69.84*	347.96 ± 79.79*	285.13 ± 63.06*
	After-treat.	16	3.72 ± 0.90*△	0.08 ± 0.07	484.55 ± 2.31*△	480.02 ± 2.35*△	411.81 ± 227.76*△

Notes: *P<0.05 *vs*. healthy control group; △P<0.05 *vs*. before treatment in the same group

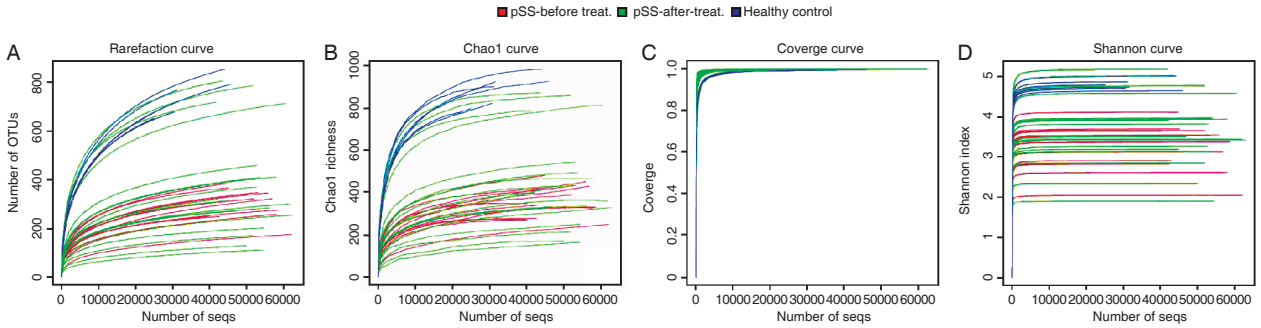

Figure 2. Dilution Curve and Shannon-Wiener Curve of Intestinal Bacteria Flora

Note: The horizontal axis is the sequence number and the vertical axis is the diversity index

matrix between pSS patients and healthy subjects, and the distance was close in pSS patients before and after treatment. Therefore, in a single sample based on OTU, the composition of the bacteria species was similar among the pSS patients, but was different before and after treatment. Furthermore, this composition after treatment was closer to that in healthy subjects than before treatment (Figure 3).

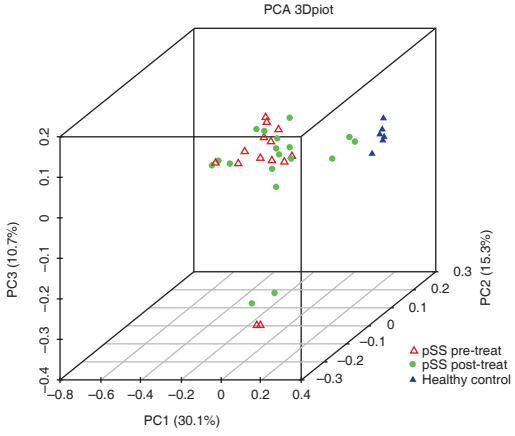

Figure 3. Analysis of Beta Diversity of Intestinal Flora

Analysis of Composition Difference of Intestinal Flora

Comparison of Flora Composition at Phylum Level

At phylum level, the proportion of some bacteria flora in pSS patients before treatment were significantly higher than that in healthy subjects (P<0.05), including *Actinobacteria*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria*. In contrast, the proportion before treatment was remarkably lower like *Bacteroidetes*, *Candidate-division-TM7*, and *Tenericums* (P<0.05). After the treatment, there was a significant decrease in the proportion of *Actinobacteria* and *Firmicutes* (P<0.05), but a significant increase in the proportion of *Candidate*-division-*TM7* (P<0.05, Table 4).

Comparison of Flora Composition at Family Level

At family level, the proportion of *Bacteroidaceae*, *Ruminococcaceae*, *Veillonellaceae*, and *Enterobacteriacea* in pSS patients before treatment were significantly higher than those in healthy subjects (P<0.05), but the proportion of *Lachnospiraceae* was significantly lower in comparision (P<0.05). The proportion of *Bacteroidaceae* and *Ruminococcaceae* after treatment were significantly decreased compared to that before treatment (P<0.05, Table 4).

Comparison of Flora Composition at Genus Level

At genus level, the proportion of some bacteria flora in pSS patients before treatment were significantly higher than that in healthy subjects (P<0.05), including *Bifidobacterium*, *Bacteroides*, *Escherichia-Shigella*, *Faecalibacterium*, and *Prevotella*. However, the proportion of *Clostridia* was significantly lower (P<0.05). After treatment, the proportion of *Clostridia* increased significantly compared to before treatment, while the proportions of *Bifidobacterium*, *Bacteroides*, *Escherichia-Shigella*, *Faecalibacterium* and *Prevotella* were significantly decreased (P<0.05). Nevertheless, when compared to

Table 4. Comparison of Flora Composition at Phylum Level, Family Level, and Genus Level (%, $\bar{x} \pm s$)

Intestinal flora	Healthy control group (6 cases)	pSS group (16 cases)	
		Before treatment	After treatment
Phylum level			
Actinobacteria	0.11 ± 0.04	1.81 ± 1.51*	0.62 ± 0.69*△
Bacteroidetes	48.31 ± 5.08	35.28 ± 16.03*	43.17 ± 13.46
Candidate-division-TM7	0.62 ± 0.36	0.00 ± 0.00*	0.40 ± 0.72△
Cyanobacteria	0.10 ± 0.19	0.00 ± 0.00	0.30 ± 0.12
Firmicutes	46.79 ± 4.79	54.50 ± 11.36*	43.99 ± 12.65△
Fusobacteria	0.00 ± 0.00	1.20 ± 1.77*	0.67 ± 0.20
Tenericutes	0.05 ± 0.03	0.00 ± 0.00*	0.02 ± 0.05
Proteobacteria	3.21 ± 1.20	8.92 ± 7.78*	7.89 ± 8.44
Family level			
Bacteroidaceae	5.19 ± 2.64	31.40 ± 12.43*	18.59 ± 14.78*△
Lachnospiraceae	28.92 ± 3.88	17.33 ± 7.23*	17.93 ± 7.90
Ruminococcaceae	14.50 ± 0.60	20.26 ± 10.90*	12.67 ± 10.70△
Veillonellaceae	0.00 ± 0.00	7.92 ± 14.02*	6.70 ± 8.60
Enterobacteriacea	0.00 ± 0.00	5.80 ± 9.06*	4.46 ± 8.39
Genus level			
Clostridia	45.19 ± 4.56	30.14 ± 11.69*	43.79 ± 10.49△
Bifidobacterium	0.00 ± 0.00	14.78 ± 1.51*	0.44 ± 0.86△
Bacteroides	5.19 ± 2.64	36.75 ± 8.37*	14.61 ± 11.25*△
Escherichia-Shigella	0.00 ± 0.00	7.22 ± 9.68*	0.22 ± 0.20△
Faecalibacterium	0.00 ± 0.00	10.62 ± 6.62*	3.25 ± 3.70△
Prevotella	2.70 ± 1.84	7.23 ± 13.33*	1.41 ± 2.38△

Notes: *P<0.05 *vs*. healthy control group; △P<0.05 *vs*. before treatment in the same group

healthy subjects, the differences were not significant (Table 4).

DISCUSSION

pSS is an autoimmune disease involving multiple systems, whose clinical symptoms are quite diverse. The most common symptoms are mouth dryness and eye dryness caused by chronic lymphocytes infiltration of exocrine glands. Most patients have extra-glandular symptoms due to system involvement, such as fatigue, joint pain/inflammation, Raynaud's phenomenon, vasculitis, leukopenia, and systemic symptoms of the liver, kidneys, lungs and nerves, which largely affect patients' quality of life and survival time.[14] ESSDAI is currently the most widely used pSS activity evaluation system in the world, which can be used to guide the evaluation of disease activity of pSS patients, and also works a useful tool for physicians to make and evaluate the therapy strategy.[15] The changes of IgG and ESR levels are related to the disease activity.[16] Therefore, combining these three indices mentioned above has important clinical significance for evaluating the pSS activity.

Recent studies have shown that pSS is caused by a combination of genetic, viral infections and abnormalities of sex hormones. These factors cause immune disorders of the body. Actually, a variety of cytokines and inflammatory mediators invade tissues such as salivary glands and lacrimal gland to stimulate the disease. Certainly, the abnormal differentiation of T helper cells (Th) and immune imbalance play an important role in the pathogenesis of pSS. Th1/Th2 cells, B cells, Th17 cells, other immune cells, and their secreted cytokines are involved in the occurrence and development of pSS.[17,18]

The bacteria in the intestines form a huge and complex ecosystem. Under healthy conditions, the intestinal flora, the host and the external environment establish a dynamic ecological balance and coordinate with the innate immune system to maintain the human health. Study has shown that intestinal microbes can activate immune cells (such as Tregs, Th17 cells, etc.) through their metabolites and other specific components, induce their directed differentiation and change the secretory function.[19] The healthy intestinal flora can maintain the immune balance and inhibit the inflammatory response. Once the intestinal flora is dysregulated, it is easy to cause the occurrence and development of inflammatory reaction. Inflammation, in turn, affects the body's immunity. It reduces immune cells and expression of cytokines, then decreases immune functions, and finally causes autoimmune diseases. Hence, the change of body's intestinal microecology is closely related to the occurrence of autoimmune disease.[20-22]

The 16SrDNA metagenomic sequencing technology is an important method to study microorganisms. It can qualitatively and quantitatively identifyy each kind of bacteria, which means it reflects the number and abundance of species in the environment based on the sequencing number of single sequence from the sequencing results. This method prevents some drawbacks existing in traditional bacterial culture techniques, such as needing living bacteria, being time-consuming, antibacterial inhibiting bacteria growth and demanding special microbial growth conditions.[23] In this study, 16SrDNA microbial sequencing technology was used to test the differences of the structure, diversity and abundance of bacteria flora in V3-V4 region of fecal samples to reflect the correlation of intestinal microecology of pSS patients with healthy subjects and disease changes. We found

that the abundance and diversity of intestinal bacteria flora were lower in pSS patients than healthy subjects. The Shannon index, Ace index, Chao index and Sobs index, which refer to the abundance and diversity of the intestinal bacteria flora, were also lower in pSS patients than healthy subjects. The Simpson index was higher in pSS patients than healthy subjects. The results demonstrate that the abundance and diversity of intestinal bacteria flora in pSS patients were lower than those in healthy subjects.

From CM point of view, pSS patients suffer from insufficient congenital endowment, yin deficiency, or dryness poison, which result in no nourishment in viscera, bones, muscles, limbs, channels and collaterals, nine orifices, and occurrence of dryness. When the disease lasts a long time, patients are subjected to qi and yin deficiency, and collaterals damage, venous stagnation and blood stasis. Clinical symptoms include mouth dryness and eye dryness, accompanied by fatigue, joint pain, constipation, dry skin, rash, less red tongue and thin pulse. CM treatment has achieved remarkable effects and has been widely applied.[24] Through a long-term investigation, our research team demonstrates that the diagnosis of pSS is primarily qi and yin deficiency and secondarily blood stasis. The YYHR can achieve good curative effects. The formula can not only relieve clinical symptoms, but also regulate immunity functions and improve the quality of life.[3,4] In non-obese diabetic mice with pSS, YYHR also reduced water consumption, increased salivary secretion, reduced the pathological damage of the submandibular gland tissue and regulated Th1/Th2 immune balance and Fas/FasL-led apoptosis.[25,26]

The results in the present study showed that treatment of YYHR significantly decreased ESSDAI scores and the levels of IgG and ESR, confirming a good therapy effect on pSS. The treatment significantly decreased the proportion of *Actinobacteria*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* of intestinal flora, but significantly increased the proportions of *Bacteroidetes*, *Teneriquetes* and *Candidate-division-TM7* at phylum level. At family level, the proportions of *Bacteroidaceae*, *Ruminococcaceae*, *Veillonellaceae*, and *Enterobacteriacea* significantly decreased, and the proportion of *Lachnospiraceae* significantly increased with YYHR treatment. At genus level, the proportions of *Bifidobacterium*, *Bacte roides*, *Escherichia-Shigella*, *Faecalibacterium*, and *Prevotella significantly* decreased, and the proportion of *Clostridia* significantly increased with YYHR treatment. We also found that there was no expression of *Euryarchaeota*, *Tenericumtes*, *Candidate-division-TM7*, *Cyanobacteria* in the intestinal flora in pSS patients with high disease activity. These indicated that with the disease improvement, the structure, abundance and diversity of the intestinal flora could be improved to healthy levels. The disease activity of pSS could also be improved by regulating the intestinal microecology.

We have found that there was intestinal microecological imbalance in pSS patients. The abundance and diversity of intestinal flora structure in pSS patients were lower than those in healthy subjects. YYHR improved the disease activity of pSS patients. It also increased the diversity of intestinal flora, significantly changed the composition and abundance of intestinal flora, and regulated the imbalance of intestinal microecology. With the disease improvement, the intestinal microecology, abundance and diversity became close to normal levels. However, how the YYHR regulates the intestinal microecology still needs further study in the future.

The present study demonstrates the existence of intestinal microecological imbalance in pSS patients, and the therapy effect of YYHR on regulating the structure, abundance and diversity of intestinal flora. However, this is only a preliminary study. The patients' conditions were mild and the sample size was small. In the future study, we will increase the sample size, enroll more pSS patients with varying degrees of disease activity, elucidate patients' changes of intestinal microecological structures and internal correlations and also deeply explore the concrete mechanism of how YYHR regulates intestinal microecology, in an attempt to provide new ideas for a comprehensive treatment of pSS.

Conflict of Interest

All authors declare that they have no any conflict of interests.

Author Contributions

Wu GL and Li TY carried out the studies, participated in collecting data, and drafted the manuscript. Lu HF and Wang Q carried out intestinal microecological detection. Chen YL and Cao H collected cases and examined specimens. All authors read and approved the final manuscript.

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